The use of Yeast Biomass and Yeast Products to Accumulate Toxic and Valuable Heavy Metals from Wastewater

J R Duncan and D Brady

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Report to the

Water Research Commission

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EXECUTIVE SUMMARY

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Background and Motivation

Water is an important though often underrated resource. Water availability and quality are of paramount importance in socio-economic growth in South Africa. It has been calculated on present rates of growth of population and industry that the supply and demand curves for potable water available in RSA will cross in the year 2020, beyond which demand will exceed supply (Water Research Needs in South Africa, P.E. Odendaal; Second Symp. Anaerobic Digestion, Bloemfontein, 1989). Metal ions can be toxic and contribute to the pollution of water, moreover they may be concentrated in certain organisms and passed on at high concentrations to humans. The importance of this problem is highlighted by the fact that humans are now the largest agent in the biogeochemical cycles of trace metals at a global scale (Nriagu and Pacyna 1988) and the toxicity of these metals now exceeds that of all radioactive and organic wastes released into the environment.

Many industrial processes produce heavy metal containing wastewaters. This represents not only a highly toxic effluent but, in the case of the mining industry, a loss of valuable metals. Removal of these metal ions is consequently vital if the wastewater is to be recycled without significant contamination of the streams/dumps into which they are discharged or loss of a potential resource.

Traditional methods of metal removal such as ion exchange and precipitation have not proved cost effective. Biotechnology based processes can,however, play a role in metal recovery. Microorganisms are known to play an active role in the solubilisation, accumulation, transport and deposition of metals in the environment (Hutchens *et al.*, 1986; Kelly *et al.* 1979). Micro-organisms are known to accumulate metals from dilute metal ion solutions and thereby concentrate them (Nakajima and Sakaguchi, 1986). This would facilitate the restoration of metal contaminated wastewater and recovery of valuable metals.

In the studies described in this report, yeast has been utilised as the micro-organism for these studies since it is a readily available waste product of alcohol based fermentation industries in South Africa and represents a relatively cheap source of biological material which requires little pre-treatment before it can be utilised for metal binding. Yeasts have also been shown, in this and other studies, to accumulate a wide range of heavy metals

from both dilute and concentrated solutions. (Norris and Kelly, 1977; Norris and Kelly, 1979; Nakajima and Sakaguchi, 1986; White and Gadd, 1987; Jones and Gadd, 1990, Rothstein and Hayes, 1956; Gadd and White, 1989). These researchers,however,did not reveal the full potential and range of yeast metal bioaccumulation, nor did they examine the possibility of using modified cells or cell components for industrial bioaccumulation purposes.

Objectives

(1) To investigate methods of utilising yeast biomass to accumulate metal ions. The preparation of the biomass should make it suitable for industrial use either for removal of toxic metals from effluents or recovery of valuable metals.

(2) To identify the components of the biomass responsible for metal accumulations and to determine whether these components are more efficient at metal accumulation than the biomass itself.

Results and Conclusions

Saccharomyces cerevisiae cells in free solution were found to be capable of accumulating Cu^{2+} in a manner that was proportional to the external Cu^{2+} concentration and inversely proportional to the concentration of biomass. The yeast biomass was also capable of accumulating other heavy metal cations such as Co^{2+}, Cd^{2+}, Hg^{+} and Cd^{2+}. The yeast biomass could be harvested after bioaccumulation by tangential filtration methods, or alternatively could be packed into hollow fibre microfilter membrane cartridges and used as a fixed-bed bioaccumulator.

By immobilising the yeast in polyacrylamide gel and packing this material into columns, Cu^{2+}, Co^{2+} or Cd^{2+} could be removed from influent aqueous solutions yielding effluents with no detectable heavy metal, until breakthrough point was reached (± 300 ml of 100 ppm solution in a 15 ml column). This capacity was hypothersized to be a function of numerous "theoretical plates of equilibrium" within the column. The immobilised biomass could be eluted with EDTA and recycled for further bioaccumulation processes with minor loss of bioaccumulation capacity.

The accumulation process in free solution or as immobilised cells was only minimally affected by temperature variations between 5 and 40°C or high ambient concentrations of sodium chloride. The accumulation process was, however,considerably affected by variations in pH, bioaccumulation being most efficient at pH 5 - 9 but becoming rapidly less so at either extreme of pH. Selection for copper resistant or tolerant yeast diminished the yeast's capacity for Cu^{2+} accumulation. For this and other reasons the development of heavy metal tolerance in yeasts was deemed to be generally counterproductive to heavy metal bioaccumulation.

Yeast cells were fractionated to permit identification of the major cell fractions and molecular components responsible for metal binding. Isolation of the yeast cell walls permitted investigation of their role in heavy metal accumulation. The use of chemical blocking and modification reagents indicated that although the amino groups of chitosan and proteins, the carboxyl groups of proteins, and the phosphate groups of phosphomannans were found to be the most efficient groups for the accumulation of copper, the hydroxyl groups of the carbohydrate polymers (glucan and mannan) had a similar overall capacity for copper accumulation owing to their predominance in the yeast cell wall,even though individually they were less effective. The outer (protein-mannan) layer of the yeast cell wall was determined to be a better Cu^{2+} chelator than the inner (chitin-glucan) layer. It appears that the physical condition of the cell wall may be more important than the individual macromolecular components of the cell wall in metal accumulation. It was apparent that the cell wall was the major, if not sole contributor to heavy metal accumulation at low ambient heavy metal concentrations.

Initial experiments on the extraction of certain cell wall components for binding experiments or removal of some components by enzymes before reacting the cell wall with metals suggests that all the major types of macromolecules found in the cell wall are important in metal binding. However, the integrity of the cell wall appears to be critical for effective metal binding.

Further experiments were carried out using a modified form of non-living yeast biomass since such a biomass could be used for metal binding without the complication of maintaining a nutrient supply to live cells and also possibly without the need to immobilise the biomass. A non-living biomass would incorporate the yeast cell wall fraction which was shown in earlier studies in this project to be the principle metal binding component of the yeast cell.

Treatment of the biomass with hot alkali yielded two biosorbents, one soluble (which could be used as a heavy metal flocculent), and an insoluble biosorbent which could be formed into a granular product to be used in fixed-bed biosorption columns. The granular biosorbent was found to accumulate a wide range of heavy metal cations (Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Ag^{2+}, Fe^{3+}, Cr^{3+}, Pb^{+}) in a semi-specific manner and could be stored in a dehydrated form indefinitely and rehydrated when required.

In conclusion, yeast cells and modified non-living yeast cells appear to provide an efficient and cost effective means of removing metal ions from solution. In an immobilised system it is possible that it can be used on a semi-continuous basis. Metal tolerance does not improve metal binding capacity. Yeast cell walls appear to be the major cell component responsible for metal binding particularly at lower ambient metal concentrations and the chemically reactive hydroxyl and carboxyl groups are of major importance in this binding. The most efficient metal binding occurs when the yeast cell wall is intact, with the individual cell wall components having a lower capacity for metal binding.

This study has consequently achieved the objectives set out in the contract and has answered the question raised therein. The study has more fully examined the potential of using yeast cells and cell wall components for metal bioaccumulation than has been previously reported. It has also extended previous studies by examining the possibility of use of a modified,non-living yeast biomass in this process, a system which has great industrial potential. While no immediate application or implementation of such a system for treatment of industrial effluent can be put forward, there is undoubted potential for such application and further studies on the mechanism of metal binding and laboratory scale effluent studies should explore this potential.

Recommendations for Further Research

(1) For the effective use of live yeast cells and/or yeast cell biomass in heavy metal accumulation an understanding of the mechanism and biochemistry of the process is essential since this will determine the types of effluents, type of biomass, metal concentrations, etc. that can be used if this process is to be industrially applicable. Further studies should consequently include detailed biochemical/mechanistic studies of yeast metal bioaccumulation.

(2) The efficiency and kinetics of metal accumulation over a range of metal concentrations should be determined since effluents are likely to be variable in their composition and metal concentrations will vary between effluents.

(3) The use of a selected system for metal removal on a continuous basis should be explored as should the desorption of metals from cells/biomass. This would again be of importance to the industrial application of this system, and in the case of the latter, would allow for recovery and/or concentration of the metal.

(4) The feasibility of using yeast cells/biomass for heavy metal removal from actual effluents needs to be investigated. Effluents of particular concern, possibly in the Eastern Cape area initially, need to be identified and the efficiency of the system in treating these effluents examined on a laboratory scale. The possible scaling-up of the process and transfer of the technology to industry will be dependant on the results of the laboratory studies.

(5) Discussions should be held on a regular basis with the groups working under Dr. P. Rose's direction on algal heavy metal accumulation, and Dr. H. Kasan's direction on the use of sewage sludges for the same purpose. It is possible that collaborative studies could arise from such discussions with the objective of identifying the most effective system for heavy metal detoxification of effluents in the South African context.

ABSTRACT

The aim of the project was to determine whether a by-product of industrial fermentations, *Saccharomyces cerevisiae*, could be utilized to bioaccumulate heavy metal cations and to partially define the mechanism of accumulation. *S. cerevisiae* cells were found to be capable of accumulating Cu^{2+} in a manner that was proportional to the external Cu^{2+} concentration and inversely proportional to the concentration of biomass. The accumulation process was only minimally affected by temperature variations between 5 and 40°C or high ambient concentrations of sodium chloride. The accumulation process was, however, considerably affected by variations in pH, bioaccumulation being most efficient at pH 5 - 9, but becoming rapidly less so at either extreme of pH. Selection for copper resistant or tolerant yeast diminished the yeast's capacity for Cu^{2+} accumulation. For this and other reasons the development of heavy metal tolerance in yeasts was deemed to be generally counterproductive to heavy metal bioaccumulation. The yeast biomass was also capable of accumulating other heavy metal cations such as Co^{2+} or Cd^{2+}.

The yeast biomass could be harvested after bioaccumulation by tangential filtration methods, or alternatively could be packed into hollow fibre microfilter membrane cartridges and used as a fixed-bed bioaccumulator.

By immobilizing the yeast in polyacrylamide gel and packing this material into columns, Cu^{2+}, Co^{2+} or Cd^{2+} could be removed from

influent aqueous solutions yielding effluents with no detectable heavy metal, until breakthrough point was reached. This capacity was hypothesized to be a function of numerous "theoretical plates of equilibrium" within the column. The immobilized biomass could be eluted with EDTA and recycled for further bioaccumulation processes with minor loss of bioaccumulation capacity.

Yeast cells were fractionated to permit identification of the major cell fractions and molecular components responsible for metal binding. Isolation of the yeast cell walls permitted investigation of their role in heavy metal accumulation. Although the amino groups of chitosan and proteins, the carboxyl groups of proteins, and the phosphate groups of phosphomannans were found to be efficient groups for the accumulation of copper, the less effective hydroxyl groups of the carbohydrate polymers (glucans and mannans) had a similar overall capacity for copper accumulation owing to their predominance in the yeast cell wall. The outer (protein-mannan) layer of the yeast cell wall was found to be a better Cu^{2+} chelator than the inner (chitin-glucan) layer. It appeared that the physical condition of the cell wall may be more important than the individual macromolecular components of the cell wall in metal accumulation. It was apparent that the cell wall was the major, if not the sole, contributor to heavy metal accumulation at low ambient heavy metal concentrations.

At higher ambient metal concentrations the cytosol and vacuole become involved in bioaccumulation. Copper and other metals caused rapid loss of 70% of the intracellular potassium, implying permeation of the plasma membrane. This was followed by a slower "leakage" of magnesium from the vacuole which paralleled Cu^{2+} accumulation, suggesting that

it may represent some form of ion-exchange.

An intracellular copper chelating agent of approximately 2 kDalton molecular mass was isolated from copper tolerant yeast. This chelator was not a metallothionein and bound relatively low molar equivalents of copper compared to those reported for metallothionein.

Treatment of the biomass with hot alkali yielded two biosorbents, one soluble (which could be used as a heavy metal flocculent), and an insoluble biosorbent which could be formed into a granular product to be used in fixed-bed biosorption columns. The granular biosorbent could accumulate a wide range of heavy metal cations in a semi-specific manner and could be stored in a dehydrated form indefinitely, and rehydrated when required.

Bioaccumulation by live algae was investigated as an alternative to yeast based processes. Various strains of algae, of which *Senedesmus* and *Selenastrum* were the most effective, were found to be capable of accumulating heavy metals such as Cu^{2+}, Pb^{2+} and Cr^{3+}.

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Glossary:

Most of the terms are derived from the Oxford pocket dictionary (6th edition) and the Oxford Concise Science Dictionary. Certain new terms are adopted from present usage by researchers in the field.

Absorption: This differs from adsorption in that the absorbed substance permeates the bulk of the absorbing substance, that is it is internalized. Absorption may be by either active or passive uptake.

Accumulation: Acquisition of something, for example metal ions. No mechanism is inferred from the term.

Adsorption: The process in which a solid holds particles of another substance to its surface. This process is usually subdivided into two processes of different orders of binding strength, i.e. Chemisorption in which a single layer of ions, atoms or molecules are attached to the adsorbent surface by chemical bonds; and Physisorption, in which the adsorbed molecules are held by the weaker van der Waal's forces. Adsorption may be considered to be a chemical or physical process rather than a biological process.

Bioaccumulation: Accumulation by metabolically active viable cells where active absorption possibly occurs.

Biosorption: as used by Volesky (1986) indicates that the accumulation is by non-viable biomass and may be a combination of adsorption and non-active absorption.

Heavy metal: There are approximately 65 elements that exhibit metallic properties, and which may be collectively termed "heavy metals". The term is imprecise but generally includes many of the transition metals (containing both lanthanide and actinide series) and some of the metals and metalloids in groups IIIB, IVB, VB and VIB of the periodic table (Sterrit and Lester, 1980; Gadd, 1990b). The term is not used with the same connotations as "heavy water" in that it does not refer to the isotope of the metal.

Abbreviations used in this text

(ATP) Adenosine triphosphate.

(CFMF) Cross-flow microfiltration.

(EDTA) Ethylenediaminetetraacetic acid

(HEPES) *N*-[2-Hydroxyethyl]piperazine-*N'*-[ethanesulphonic acid].

(MRA) Metal recovery agent.

(MT) Metallothionein.

(PAGE) Polyacylamide electrophoresis.

(PIPES) Piperazine-*N,N'*-bis[2-ethanesulphonic acid]

(ppb) Parts per billion.

(ppm) Parts per million.

(PTM) Transmembrane pressure.

(RO) Reverse osmosis.

(rpm) Rotations per minute.

(TEMED) (*N,N,N',N'*)-tetramethylethylenediamine.

(TMAH) Tetramethylammonium hydroxide.

(UF) Ultrafiltration.

GENERAL INTRODUCTION

1. GENERAL INTRODUCTION

1.1 WATER: A RESOURCE

Water is an important though often underrated resource. It is vital for both domestic and industrial purposes. In industry water may be used not only in cooling and the removal of wastes, but also in the product itself, such as beverages and processed foodstuffs. The United Nations designated the 1980s as "The International Drinking Water Supply and Sanitation Decade," in recognition of the importance of high quality water in the maintenance of a healthy population (Dean and Lund, 1981).

Water availability and quality are of paramount importance to the socio-economic growth in South Africa. The average rainfall in the Republic of South Africa is 483 mm per annum, which is far below the global average of 860 mm per annum (Odendaal, 1989). With low rainfall run-off becomes a far bigger percentage, and losses are increased by high levels of evaporation in the R.S.A. due to the warm climate. Moreover irregular rivers, which are common in R.S.A., are very sensitive to pollution which tends to build up in them. It has been calculated that the supply and demand curves for potable water available in the R.S.A. will cross in the year 2020, and beyond that demand will exceed supply (Odendaal, 1989). This country is one of the few in the world facing so immediate a problem, the R.S.A. therefore requires its' own research program to solve these problems as this area of technology has not been adequately developed elsewhere.

1.2 METALS AS POLLUTANTS

With such a high demand for limited quantities of potable water it is necessary to prevent or at least limit its tainting with pollutants. One commonly encountered group of pollutants are toxic metals. Since the advent of the industrial revolution there has been a trend of processing increasing tonnage of metals for manufacture of products. There are numerous opportunities for metal release into the environment during the sequential mining, refining and final processing of a metal. This problem is aggravated by the habits of the modern "throw-away society" which encourages built-in obsolescence in manufactured articles, and yet has only a very limited infrastructure for recycling these materials.

Metals can be extremely toxic and therefore become a health hazard to humans and the environment if not handled carefully (a full review is presented in Appendix 2). Another salient point is that metals are expensive to locate, mine and refine. If metal could be reclaimed from waste or if losses during each step in metal processing could be reduced, then the opportunity is presented to produce cheaper goods with a higher profit margin. On a less mercantile note, metals are considered to be a non-renewable resource and should be managed with care. For South Africa in particular this area of research is of singular importance. South Africa is a major metal ore mining and refining country and contains a substantial percentage of the world's known valuable metal resources. If the output of the mines could be increased and efficiency improved even by a small percentage, then the financial rewards could be vast.

1.3 BIOLOGICAL METAL RECOVERY: A POSSIBLE SOLUTION

Biotechnology is a field of study and activity which overlaps and combines the knowledge, techniques and resources of the biological, chemical, physical and engineering sciences, (Lakshmanan, 1986) to yield tremendous new possibilities. Biotechnological approaches to metal recovery are now considered as practical options to traditional metallurgical techniques for reasons that are stated below.

Microorganisms are known to play an important role in the solublization, accumulation, transport and deposition of metals in the environment (Hutchins *et al*, 1986; Kelly *et al*, 1979). Living organisms must be able to cope and utilize the inorganic world around them in order to survive and flourish, and it is not surprising that the global microbial mineral recycling process began as soon as the first life forms appeared on earth. Calculations suggest that in the intervening time the total biomass has recycled inorganic ions of a quantity that is equivalent to the mass of the earth's crust (Beveridge, 1986).

Microbial fossils or remnants are often associated with high metal concentrations in ancient geological horizons. The process of deposition of these metals has not been elucidated and the association of these mineral deposits with microbial remnants strongly suggests the possibility of biological origins for the mineral deposits (Beveridge and Murray, 1976). Branded ironstone formations, which are composed of ancient iron oxide deposits in rock strata, are thought to be the result of microbial action, *i.e.* oxidation of the environment by cyanobacteria. The strata of gold located in the Vaal Reef may be the

result of prehistoric deposition of gold ions chelated to biological materials in the sediments of prehistoric river beds (Davidson, 1990). Microbial action has also been implicated in the formation of ferro-manganese nodules which are to be found dispersed on the bed of the ocean (Gadd, 1990b). The manipulation of this biological facility for mineral interaction, biohydrometallurgy, could yield numerous potential new technologies.

1.3.1 Biological Metal Mobilization: Bioleaching

One of the most intensively studied research topics in the area of biohydrometallurgy has been the use of bacteria in leaching metals from low grade metal ores (such as those found in mine dumps where other extraction procedures would be inefficient). The principles, methods and applications of bacterial mine dump leaching have been well reviewed (Hutchins *et al*, 1986; Kelly *et al*, 1979; Brierley, 1982). Briefly re-stated, this leaching may involve direct enzymatic oxidation of the substrate at the cell surface or an alternative process referred to as either bacterially assisted leaching or indirect leaching. Indirect leaching utilizes bacteria such as *Thiobacillus ferrooxidans*, an acidophilic bacterium which obtains energy from the oxidation of either iron or sulphur. It oxidizes soluble ferrous (divalent) iron into ferric (trivalent) iron (a strong oxidizing agent) which in turn reacts with other metals in the substrate, releasing them in an oxidized, soluble form. This reaction yields ferrous iron which may again be oxidized by bacteria in a further cycle. Mine dump leaching by bacteria is now an established industrial practice. These bacteria are capable of leaching copper from covellite (CuS) and iron from pyrite, chalcopyrite and pentlandite (Brierley, 1982).

Bioleaching mobilizes metals from deposits and therefore reduces the environmental pollution caused by these waste products (Bosecker, 1986) but fails to offer a method of accumulating these metals; indeed, metal accumulation may be inhibitory to the bioleaching process. To accumulate metals using microorganisms, other techniques are required.

1.3.2 Biological Metal Immobilization: Bioaccumulation

A less thoroughly investigated area of biohydrometallurgy is the use of microorganisms to recover metal ions from wastewaters. Microorganisms are known to accumulate metals from dilute metal ion solutions and thereby concentrate them (Nakajima and Sakaguchi, 1986). This would facilitate the restoration of metal-contaminated wastewater and recovery of valuable metals. This is the fundamental interest of the present research.

In spite of the recent interest in bioaccumulation of metals, industrial microbial metal bioaccumulation processes are rare (Tobin *et al*, 1990). The proceedings of a recent conference (IX Int. Symp. Biohydrometallurgy) on all aspects of biohydrometallurgy indicated that most of the bioaccumulation work was still firmly on the laboratory bench. A part of the reason for this is that industrial processes must be predictable to succeed, even if it is technically and economically feasible. If a problem in the process arises then it must be relatively easy to find its cause and correct it before time and money are lost. It has been stated by Tobin *et al* (1990), however, that our present understanding of the metal binding mechanisms is incomplete and models presented are diverse and often difficult to reconcile. This situation must be remedied if industrial bio-accumulation is to succeed.

1.4 RESEARCH AIMS

The central aim of the present work is to determine how yeast biomass can be utilized to accumulate metal cations from dilute aqueous solutions. The yeast *Saccharomyces cerevisiae* has been chosen because it can be obtained relatively cheaply as a by-product of certain fermentation industries, and in quantities sufficient for industrial scale metal accumulation. The ready availability of an inexpensive raw material which has been proved to be effective would undoubtedly improve the chances of microbial metal bioaccumulation being adopted by industry. Moreover, the capacity of yeasts to accumulate significant amounts of metals has been known for some time; for instance cobalt-tolerant *S. cerevisiae* was reported during the middle of this century (Nickerson and Zerahn, 1949; Perlman and O'Brien, 1954). Later studies demonstrated that yeasts are able to accumulate a wide range of metal ions (Norris and Kelly, 1977, 1979; Nakajima and Sakaguchi, 1986). Thirdly, the structure and metabolism of *S. cerevisiae* has been well studied owing to its economic importance, and therefore a large data base is available on yeast which does not exist for many other species of microorganisms.

Although a wide range of heavy metals have been used in this work, copper (II) has been chosen as the focal metal cation because that it is fairly representative of toxic heavy metals in general, and that it forms a blue-green hue on biomass during accumulation, allowing for visual confirmation of both analytical results and uniformity of permeation in fixed-bed biomass columns.

A secondary aim of this research is to elucidate the mechanisms of metal accumulation by *S. cerevisiae*. It is hoped that a detailed knowledge of the metal accumulation processes will enable more efficient usage of the yeast to obtain either greater accumulation yields and/ or more selective accumulation.

A schematic overview of the research areas investigated is presented in figure 1.

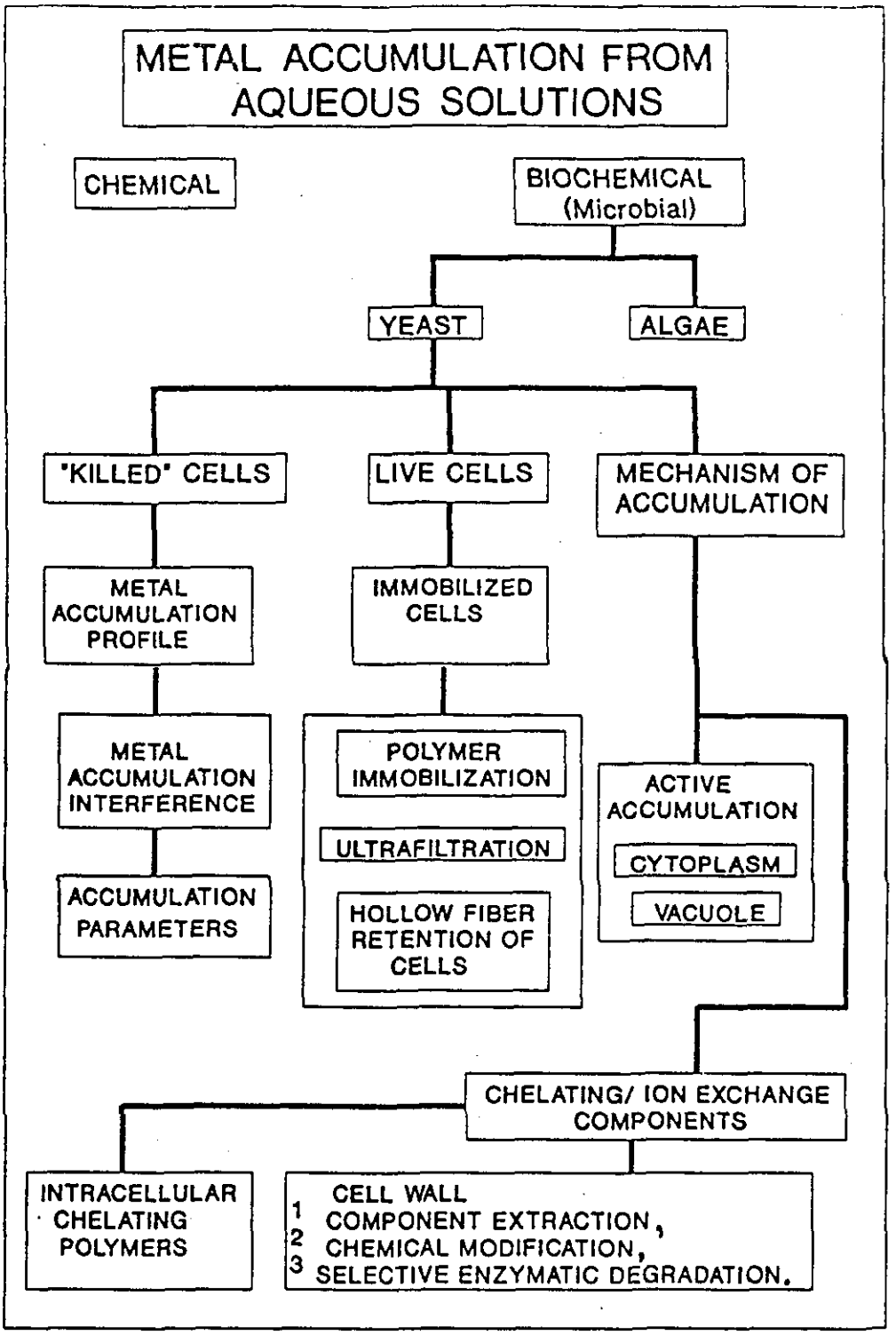


Figure 1: Schematic flow diagram of areas of research pursued within the frame-work of the present study and their inter-relationships.

PART 1: BIOACCUMULATION

2. BIOACCUMULATION OF METAL CATIONS BY VIABLE YEAST

2.1 INTRODUCTION

Microorganisms accumulate metals by a number of different processes such as uptake by transport, biosorption to cell walls and entrapment in extracellular capsules, precipitation, and oxidation-reduction reactions (Lundgren *et al*, 1986; Gadd, 1990a, 1990b; Macaskie and Dean, 1984). Some or all of these processes may be invoked by viable (living) microorganisms to accumulate or immobilize soluble metal ions. Microbes may also serve other functions in water treatment simultaneously with metal removal; this would be fortunate and could aid the economic or technical viability of the process.

A major problem related to bioaccumulation is that cells are prone to toxins that may be present in wastewaters, including the heavy metals that are the subject of this study. This problem can be avoided by the separation of the microbial growth phase and the metal accumulation step. This in turn suggests that any industrial microbial biomass can be utilized even if the biomass is not tolerant to heavy metals. Hence the choice of *Saccharomyces cerevisiae* biomass as a bioaccumulation agent is readily justified.

Alternative choices are available, however, and should not be overlooked. Many filamentous fungi are used in production of antibiotics and enzymes and are therefore available as a waste product of these industries. The waste could be used in bioaccumulation

processes. An example of this is the filamentous fungus *Rhizopus arrhizus* which was shown to accumulate cadmium (Lewis and Kiff, 1988).

2.1.1 Bioaccumulation of Metal Cations by Yeast:

S. cerevisiae can accumulate heavy metals, such as Co^{2+} and Cd^{2+}, *via* two distinct processes. There is an initial rapid accumulation step which is metabolism- and temperature-independent and is thought to involve cation binding to the cell surface. This step is followed by a second process which is metabolism-dependent, much slower, and can accumulate larger quantities of cation than the first process. This second process is believed to involve cation internalization into the cell (Norris and Kelly, 1977). The uptake system which allows for accumulation of cobalt and cadmium cations appears to be a general one with only limited specificity, since competition for uptake of cations occurs (Norris and Kelly, 1977). Further investigations proved that yeasts are capable of accumulating other cations such as copper, nickel and manganese and are superior metal accumulators compared to certain bacteria (Norris and Kelly, 1979). Although an alternative study of a wider range of microorganisms showed many bacteria to be superior heavy metal cation accumulators compared to yeasts (Nakajima and Sakaguchi, 1986), *S. cerevisiae* exhibited the highest overall capacity for metal ion uptake from mixed cation solutions among the yeasts examined. *S. cerevisiae* was one of a range of fungi that were shown to accumulate cadmium (Cd^{2+}) cations as well as Cu^{2+}, Zn^{2+}, Pb^{2+} and Co^{2+}, by Huang *et al* (1988), who believed the major accumulation mechanism to be adsorption.

S. cerevisiae has been demonstrated to accumulate uranium from the wastewater of the nuclear fuel industry (Shumate *et al*, 1978). Uranium

uptake by the yeast increased with increase in temperature between 25°C and 40°C, and was similarly dependent on increased uranium concentration. The uranyl ion (UO_2^{2+}) may bind to the cell surface phosphate groups and possibly carboxyl groups; no uranium was accumulated endogenously (Rothstein and Hayes, 1956; Strandberg *et al*, 1981). Electron microscopy and energy dispersive X-ray analyses showed that uranium accumulated in needle-like shapes in a layer on the exterior of *S. cerevisiae* cells (Strandberg *et al*, 1981). At low ambient pH (< 2) yeasts are also capable of binding another metal which has a radioactive isotope, *viz.* thorium. The quantity of thorium uptake varied with the growth medium used to produce the biomass (Gadd and White, 1989).

Zinc cation bioaccumulation by the fungus *Candida utilis* is similar in some aspects to the general metal cation bioaccumulation processes of *S. cerevisiae*. Initially Zn^{2+} accumulation by *C. utilis* is rapid, energy- and temperature-independent, and probably represents binding to the cell surface (Failla *et al*, 1976). The second process of Zn^{2+} uptake by *C. utilis* requires intact membranes (Failla *et al*, 1976) and the system of accumulation exhibited saturation kinetics. However, unlike the bioaccumulation of heavy metals by *S. cerevisiae* this process was relatively specific as various other ions (Ca^{2+}, Cr^{3+}, Mn^{2+}, Co^{2+} or Cu^{2+}) did not compete with, inhibit, or enhance the zinc uptake process. Intracellular uptake was dependent on metabolic energy, pH and temperature, and was capable of accumulating far greater quantities of Zn^{2+} than the initial binding process. The zinc was taken into a non-exchangeable pool. *C. utilis* accumulated zinc internally only during the lag phase and the latter half of the exponential phase; however,by far the greatest uptake per mass of

cells occurred during the initial log phase (Failla *et al*, 1976, 1977). The data presented indicated that *de novo* protein synthesis was required for membrane translocation of Zn^{2+} by the cells of *C. utilis*.

Yeasts also have the facility to precipitate metals as sulphides in and around cell walls, and colonies may assume a dark brown colour in the presence of copper (Ashida, cited by Gadd, 1990b). Other species of microbes precipitate metals at the cell surface by oxidation reactions, while some have been noted to precipitate metals as phosphates by means of a cell-bound phosphatase (Gadd, 1990b).

2.1.2 Applications of Bioaccumulation:

(a) Radionucleotide recovery:

There is interest in the use of microbial based processes in the nuclear fission industry (Francis, 1990). For instance *S. cerevisiae* accumulates uranium, cesium and radium, primarily by means of surface ion exchange (McCabe, 1990). These metals are all known for the radioactivity of their isotopes. The uptake of uranium by *S. cerevisiae* was slow compared to that by *Pseudomonas aerugenosa* (Shumate *et al*, 1978).

Galun *et al* (1983, 1984, 1987) noted that the common fungus *Penicillium* could effectively accumulate 90% of the uranium from a 1 ppm solution, and was effective at much higher concentrations of the uranyl ion as well. Recycling the biomass with no loss of accumulation capacity was possible using acidic carbonate solutions which removed up to 99% of the uranium from the biomass. In field experiments *Aspergillus ochraceus* was found to accumulate uranium from mining wastewater even when it was present at very low ambient concentrations (Berthelin *et al*, 1991).

Rhizopus arrhizus has been shown to be more effective in accumulating uranium and thorium from wastewater solutions than a commercial ion exchange resin or activated carbon (Tsezos and Volesky, 1981). Again a carbonate solution, this time slightly alkaline, was capable of eluting the uranium from the biomass without significantly damaging the biomass; this was attributed to the high affinity of the carbonate ion for uranium (Tsezos, 1984). Pilot metal bioaccumulation studies have been undertaken with this microorganism (Tzesos, 1991).

Shumate *et al* (1980) utilized a mixed culture of denitrifying bacteria to simultaneously accumulate nitrogen and uranium from nuclear materials processing wastewater. The bacteria were grown as biofilms on anthracite particles at 25°C and placed in a columnar contactor where particles of the biosorbent settled and the upward flow of the liquid allowed for counter-current accumulation.

Streptomyces bacteria have recently been employed in the bioaccumulation of uranium from uranium mining leachate (Glombitza *et al*, 1991). The uranium could be eluted from the biomass, and the biomass reused.

(b) Treatment of acidic mine drainage:

Chemical reactions between oxygen, ground water, and any of a number of sulphide-containing minerals (such as pyrite, FeS_2) may lead to the formation of acid mine water. As the name implies the water is a dilute sulphuric acid solution, and it has the capacity to mobilize metals from the ore. The problem is pervasive and abandoned mines are a principle cause of this acidification, with concomitant heavy metal pollution (Unz and Dietz, 1986). Bioaccumulation could possibly be

used to alleviate this problem. An obvious added bonus in applying bioaccumlation in this case is that the accumulated metal may be commercially valuable.

There has been interest in the possible use of *Thiobacillus ferrooxidans* to desulphurize coal fines during flotation procedures. The process was found to work, but the mechanism was very different from that envisaged initially. There was none of the expected sulphuric acid production and the reaction took only 10 seconds to occur. It was later found that *S. cerevisiae* was equally competent in suppressing pyrite flotation, and disrupted cells functioned better than whole cells (Townsley and Atkins, 1986). The speed of reaction and the fact that no enzymatic processes were necessary suggests that the reaction may be a surface binding-related system, possibly related to a bioaccumulation mechanism.

(c) Treatment of wastewaters:

Activated sludge is a biological waste treatment system commonly utilized by wastewater treatment facilities to oxidize, and thereby degrade, sewage. The oxidation is caused by the action of a mixture of bacteria. Activated sludge processes are among the best commercial methods presently used in metal removal from aqueous streams; a combination of flocculation and settling is employed to separate metals (Lundgren *et al*, 1986). Activated sludge treatment processes are capable of removing large quantities of heavy metal cations from solution (Oliver and Cosgrove, 1974). A bacterium commonly found in sludges, *Zoogloea ramigera*, produces an extracellular acidic polysaccharide which can be removed from living cells by certain culture techniques, collected, and used to complex heavy metals

(Norberg and Rydin, 1984). The material may be regenerated and reused.

However, the activated sludge system is not totally effective, and as the majority of metallic elements pass through the water treatment process and insoluble particles settle, the proportion of dissolved- to total-metal increases. For metals such as Cu, Mn, Ni, and Zn, almost all of the metal in the final effluent is present in the dissolved form, and increased duration of settling or the addition of flocculating agents would be ineffectual in precipitiating these metals from the effluent. Any proposed biological tertiary treatment process must therefore be efficient at removing dissolved metals from the effluent (Oliver and Cosgrove, 1974).

Metal toxicity to water purification plants is a pervasive problem. Heavy metals can have a profound effect on the biological processes in activated sludge systems (Tyagi, 1985). It has been suggested on the basis of experimental results that the addition of yeast extract into the sludge could have a beneficial chelating effect which would reduce metal toxicity (Callander and Barford, 1983).

2.1.3 Research Aims:

The aim of this section of the present research was to investigate the process of heavy metal bioaccumulation by suspensions of viable yeast (*S. cerevisiae*) and determine how this process is altered by the imposition of various extracellular environments.

2.2 MATERIALS AND METHODS

2.2.1 Preparation of Solutions:

To limit metal contamination, all aqueous solutions were prepared with ultra-pure water (purified by Millipore Milli-Q purification system). All glassware used was of borosilicate glass which has relatively low metal cation binding properties. Glassware was prepared for use by washing with detergent, rinsing, and then heating in a 1 : 1 solution of 55% nitric acid : water solution (80°C, 12 hours), washed with ultra-pure water, and heat dried. Metal analyses were carried out by flame atomic absorption spectroscopy according to the methods of Greenberg *et al*, (1980) using a Varian Techtron 1000 atomic absorption spectrophotometer (see Appendix 3 for metal standards and Appendix 4 for depiction of the instrument used).

2.2.2 Bioaccumulation of Heavy Metal Cations:

The methodology used was similar to that of Norris and Kelly (1977). *S. cerevisiae* cells, obtained from commercial suppliers, were washed twice with ultra-pure water after centrifugation at 1 000 x g for 10 min., and resuspended in 5 mmol.dm^{-3} piperazine-*N*,*N*'-bis(2-ethanesulphonic acid) buffer (PIPES (Sigma Co.); Good *et al*, 1966) which had been adjusted to pH 6.5 with tetramethylammonium hydroxide (TMAH (Sigma Co.), see Appendix 3). This buffer was chosen for its negligible metal-chelating properties. An absorption/cell dry mass standard curve was developed by measuring the absorptions of cell suspensions at 540 nm using a UV-visible light spectrophotometer (Bausch and Lomb Spectronic 1001) and relating this to the dry mass of the suspensions after drying at 80°C for 24 hours (Appendix 3). Cell

suspensions to be used in bioaccumulation assays were adjusted to approximately 0.4 mg dry mass.cm^{-3} or 1.0 mg dry mass.cm^{-3} by dilution with buffer, with reference to their absorption at 540 nm according to this standard curve.

Duplicate yeast cell suspensions (48.5 cm^3) were shaken in 250 cm^3 Erlenmeyer flasks on a reciprocal shaker at 25°C. Aliquots (0.5 cm^3) of either ultra-pure water or 1 mmol.dm^{-3} dextrose was added to the flasks 10 minutes before the addition of 1 cm^3 of a metal salt solution (at a concentration of 50 x the required final concentration of 200 μmol.dm^{-3}). Samples (2 cm^3) were taken, using a syringe, at intervals after metal salt addition and filtered (0.45 μm, 25 mm diameter Millipore HA membrane filters in reuseable Millipore filter holders). Filters were washed immediately with 5 cm^3 PIPES buffer, removed from the holders, and transferred to glass centrifuge tubes.

To each centrifuge tube containing a filter, 0.2 cm^3 of 55% HNO_3 (analytical grade, AECI) was added, and the tubes were incubated in a boiling water bath for 60 minutes to release cell-associated metal ions. Samples were made up to 4 cm^3 with ultra-pure water, centrifuged (1 000 x g, 10 min.) to remove any undigested particulate matter, and both the supernatants and filtrates were analysed for metal content by flame atomic absorption spectrophotometry.

2.2.3 Relationship of Copper Bioaccumulation to the Ambient Copper Concentration:

Cell suspensions were incubated for 60 minutes at 25°C (as above, except a total volume of 20 cm^3 was used) in one of a range of buffered $CuCl_2$ solutions of various concentrations to determine the

effect of copper concentration on copper bioaccumulation.

2.2.4 Effect of pH on Copper Bioaccumulation:

Ambient pH was modified by addition of HCl or tetramethylammonium hydroxide to cell suspensions. No buffer was included but 5 mmol.dm^{-3} sorbitol (final concentration) was included to maintain osmotic strength. After incubation with 200 μmol.dm^{-3} $CuCl_2$ the cell suspension was centrifuged at 3 000 x g (10 min.) and the supernatant was analysed for copper by flame atomic absorption spectroscopy.

2.2.5 Effect of Temperature on Copper Bioaccumulation:

This simply involved the maintenance of the buffered cell suspension in the presence of buffered $CuCl_2$ (200 μmol.dm^{-3}), at set temperatures using heated water baths. Yeast cell suspensions were brought up to the set temperatures before addition of the copper chloride solution. After one hour samples were taken, filtered, and assayed as in the metal bioaccumulation assay above.

2.2.6 Effect of Ionic Strength on Copper Bioaccumulation:

Cell suspensions were incubated with one of a range of sodium chloride solutions in buffer to determine the effect of ionic strength on the bioaccumulation of added copper (200 μmol.dm^{-3} $CuCl_2$).

2.2.7 Visualisation of the Cell-Copper Interaction using Fluorescent Dye:

A cell suspension (9 cm^3 of 0.1 mg.cm^{-3} wet mass in buffer) was incubated at 25°C with 1 cm^3 of $CuCl_2$ solution (10 mmol.dm^{-3}) or H_2O for five minutes. To this cell suspension was added 1 drop of a 1mg.cm^{-3} 2-7-Dichloro-fluorescene (BDH, England) solution. Slide

preparations of the cell suspension were then viewed under phase contrast microscopy and subsequently under phase contrast microscopy in fluorescence mode (Zeiss Phase-Contrast Microscope, Neofluar Ph 3, Neofluar 100/1.3 oil (160/-) lens using Zeiss *Immersions oël* 518c). A Zeiss UV light source with a blue filter was used during microscopic observations. Photographs were taken using a Zeiss MC 63 Photo Timer to control exposure.

2.3 RESULTS:

2.3.1 Bioaccumulation of Heavy Metal Cations:

Biomass of the yeast *S. cerevisiae* accumulated the divalent cations of copper, cadmium and cobalt. The accumulation of copper over time can be seen in figure 2.1. Metal accumulation is reported in nmol metal accumulated per mg dry mass of yeast biomass. The shape of the bioaccumulation curve was dependent upon the ratio of cation to biomass concentration. With higher biomass concentrations there was no observable second, slower, copper bioaccumulation process. In the majority of later experiments 0.4 mg dry mass yeast per cm^3 was used in assays, as this mass exhibited both types of accumulation at the concentrations of copper used.

The addition of glucose to the cell suspension did not appear to enhance Cu^{2+}, Co^{2+} or Cd^{2+} bioaccumulation (figures 2.2, 2.3 and 2.4). The bioaccumulation curves of yeast suspensions for the three cations appeared similar at these concentrations (figure 2.5).

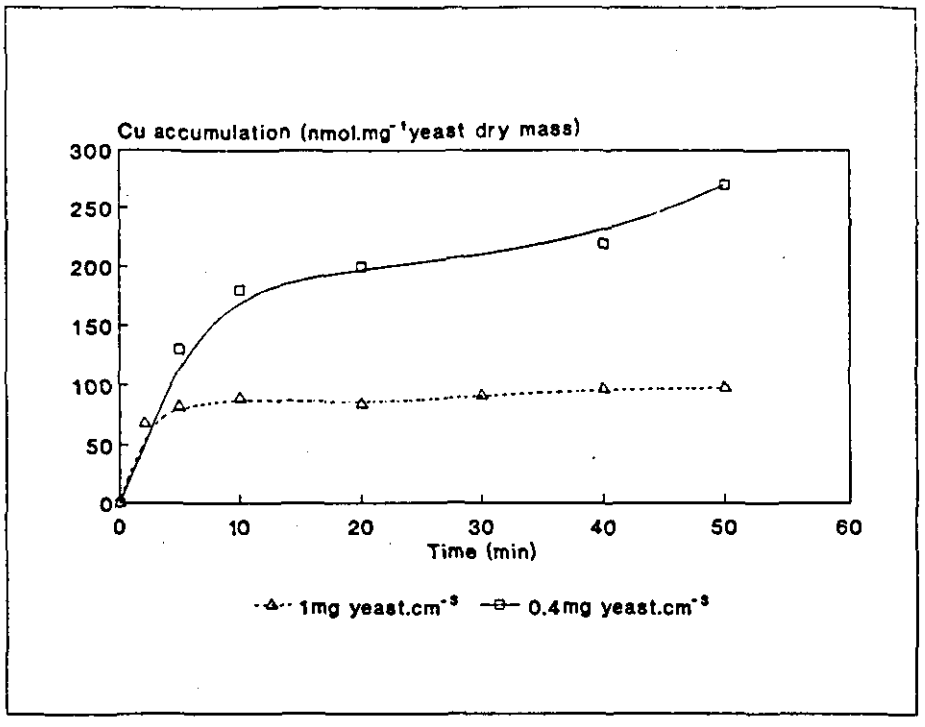


Figure 2.1: Copper bioaccumulation by *S. cerevisiae* cells at two biomass concentrations, 1 mg.cm^{-3} and 0.4 mg.cm^{-3} (dry mass per volume).

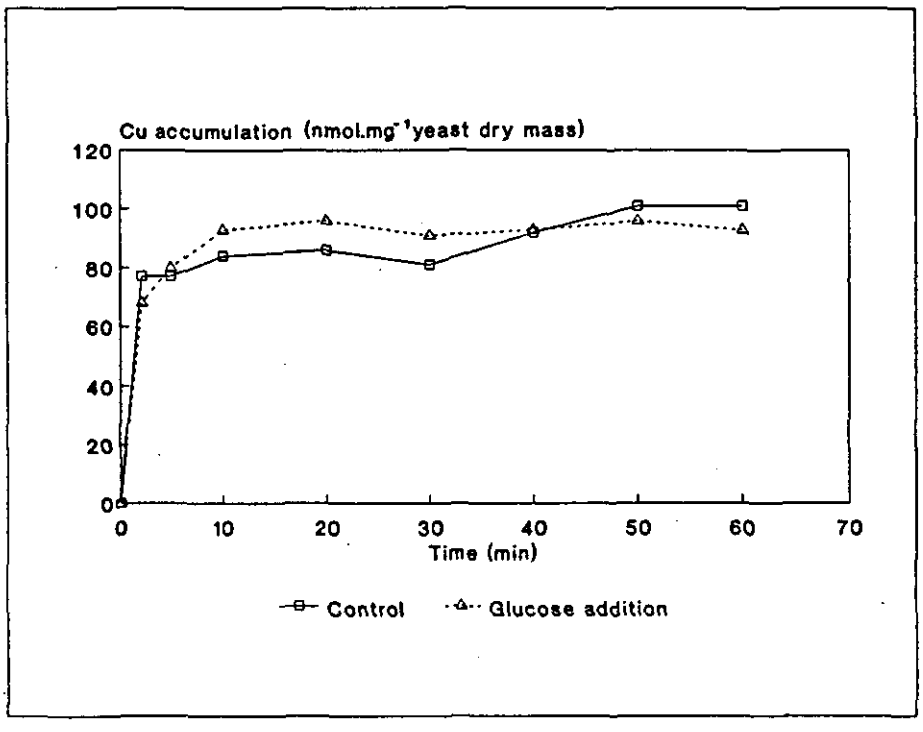


Figure 2.2: Copper bioaccumulation by *S. cerevisiae* suspensions, with and without glucose addition. Cell concentration was 1 mg.cm^{-3} (dry mass per volume).

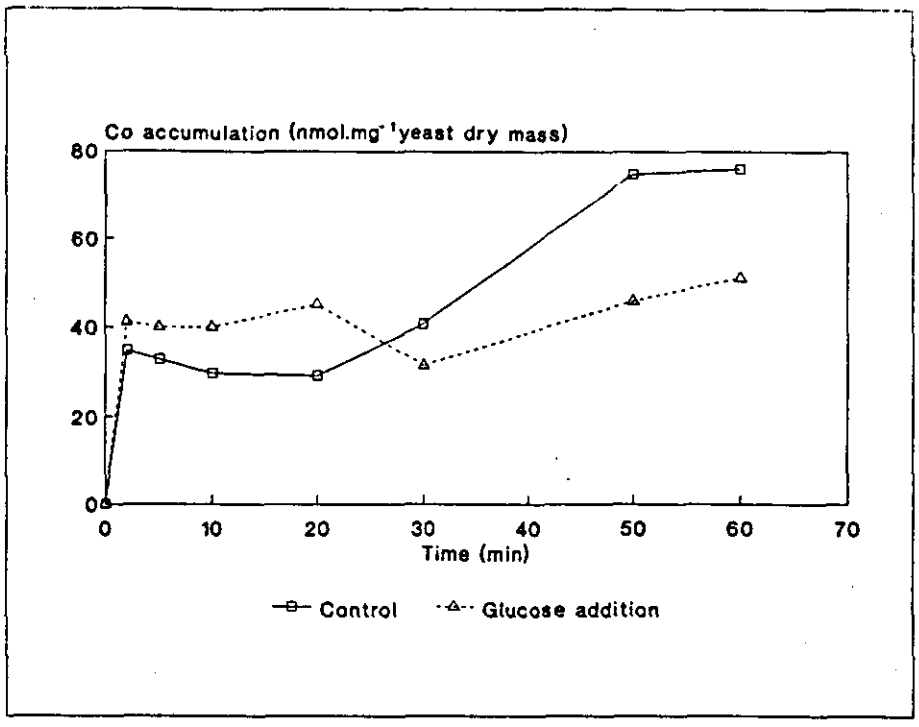


Figure 2.3: Cobalt bioaccumulation by *S. cerevisiae* suspensions, with and without glucose addition. Cell concentration was 1 mg.cm^{-3} (dry mass per volume).

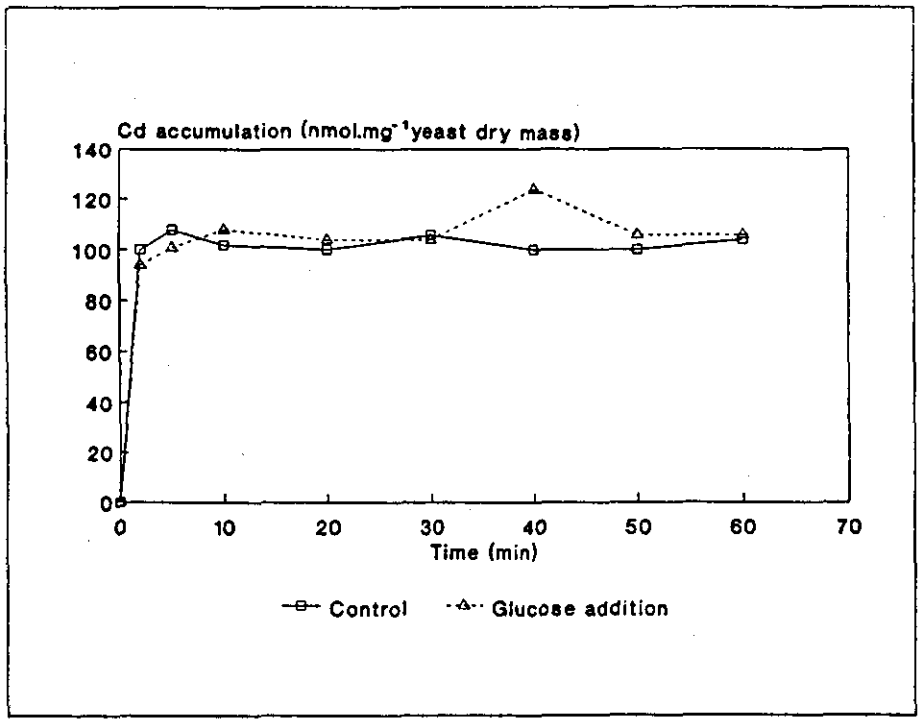


Figure 2.4: Cadmium bioaccumulation by *S. cerevisiae* suspensions, with and without glucose addition. Cell concentration was 1 mg.cm^{-3} (dry mass per volume).

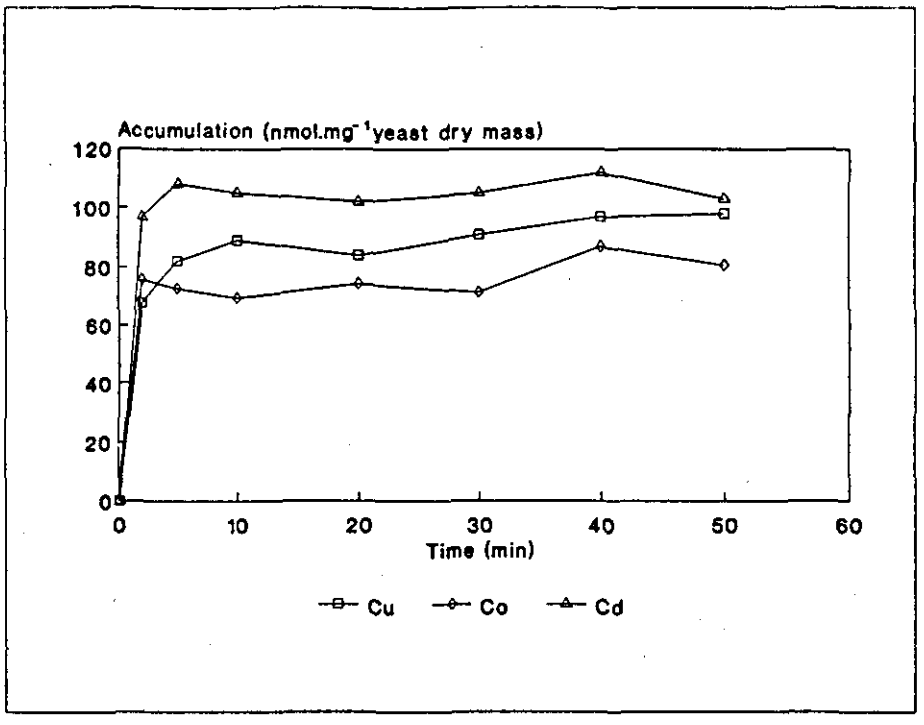


Figure 2.5: Bioaccumulation of metal ions by yeast suspensions. In each case cell concentration was 1 $mg.cm^{-3}$ (dry mass per volume).

There do not appear to be active accumulation mechanisms for Cu^{2+}, Co^{2+} or Cd^{2+} in this strain of yeast since at low metal to biomass ratios there was negligible accumulation of residual free metal after metal binding to the cell wall occurred. Moreover, the addition of a metabolizable substrate (glucose) did not facilitate or stimulate any such mechanism.

2.3.2 Relationship of Copper Bioaccumulation to the Ambient Copper Concentration:

The results presented in figure 2.1 indicate that copper cation accumulation by yeast was dependent on the ratio of external free metal ion concentration to the available biomass. The results of more comprehensive experiments comparing metal bioaccumulation to ambient

metal concentration (presented in table 2.1 and figure 2.6) agree with this suggestion. External metal concentrations affect both the metal binding equilibrium and the concentration gradient across the cell membranes. This means that over the cation concentration range investigated the percentage accumulation remained similar for all concentrations. This concept will be further developed in chapter 4.

TABLE 2.1: Copper accumulation with varying ambient copper concentration.

Cu concentration ($\mu mol.dm^{-3}$)	Total Cu (μmol)	Total Cu accumulated (μmol)	% accumulation
0	0	0	-
50	1	0.56	56
100	2	1.00	50
150	3	1.67	56
200	4	2.06	52
300	6	3.48	58
400	8	4.72	59
500	10	5.44	54

Average percentage accumulation was 55%. The concentration of biomss used was 0.4 $mg.cm^{-3}$ (dry mass per volume).

A Scatchard plot is a graphical representation of accumulation data which permits an estimation of the affinity between the accumulator and metal ions (its derivation and implications are explained in section 6.3). The overall affinity of yeast cells for Cu^{2+} was found to be relatively low according to a Scatchard plot of Cu^{2+} accumulation (figure 2.7). The amount of bioaccumulation would therefore be subject to change with relatively minor variations in external Cu^{2+} concentrations. The affinity of whole cells was much lower than that seen for the isolated yeast cell walls (see section 6.3).

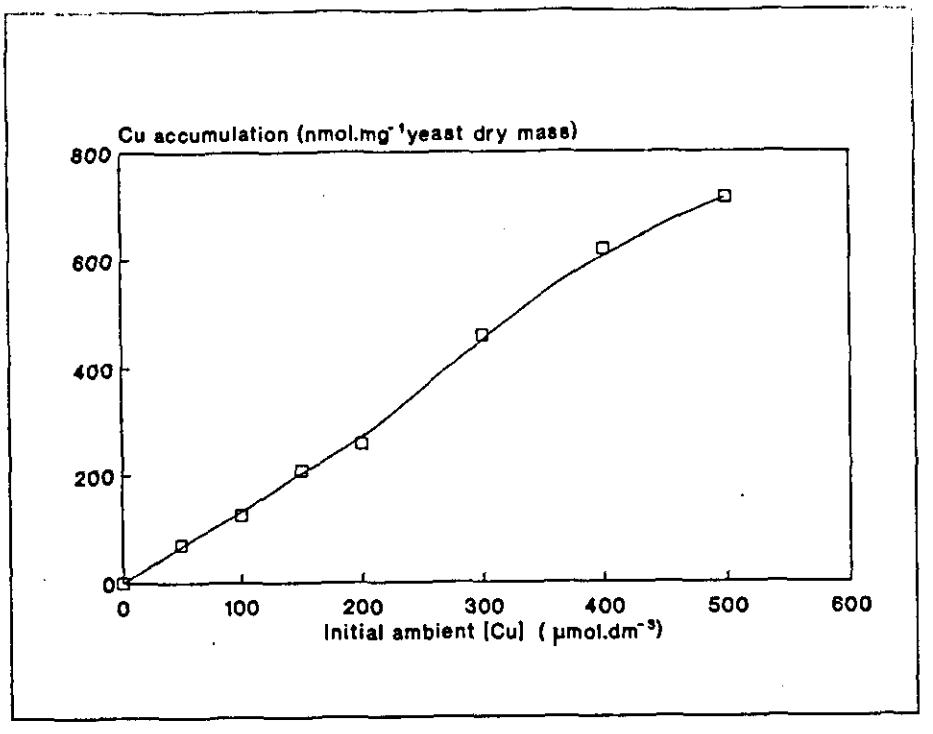


Figure 2.6: Variation of copper bioaccumulation with varying ambient extracellular copper concentration.

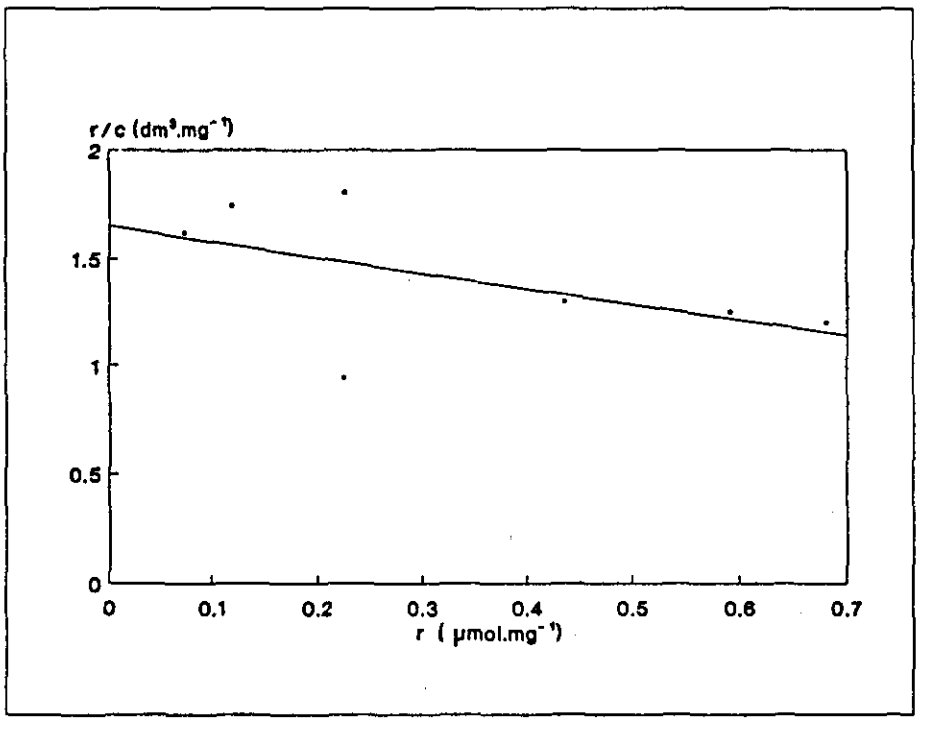


Figure 2.7: Scatchard plot of the bioaccumulation of copper by yeast cell suspensions. This figure was derived from the results presented in table 2.1 and figure 2.6. The steeper the slope of the graph the higher the affinity of the accumulator for the ion.

2.3.3 Effect of pH on Copper Bioaccumulation:

Ambient pH is likely to be a major factor in the quantity of metal ion bioaccumulation owing to cation competition effects with the hydronium ion (H^+); the results presented in figure 2.8 support this assumption. The pH region of maximum Cu^{2+} accumulation was pH 5 - 9, with rapid decreases in Cu^{2+} accumulation at either extreme of the pH range, particularly towards the acid region. In figure 2.9 a comparison of copper bioaccumulation in the presence of PIPES and Tris buffers is presented. There was little difference in bioaccumulation quantities in the different buffers at these pHs, indicating that the buffer choice is not critical for the bioaccumulation process.

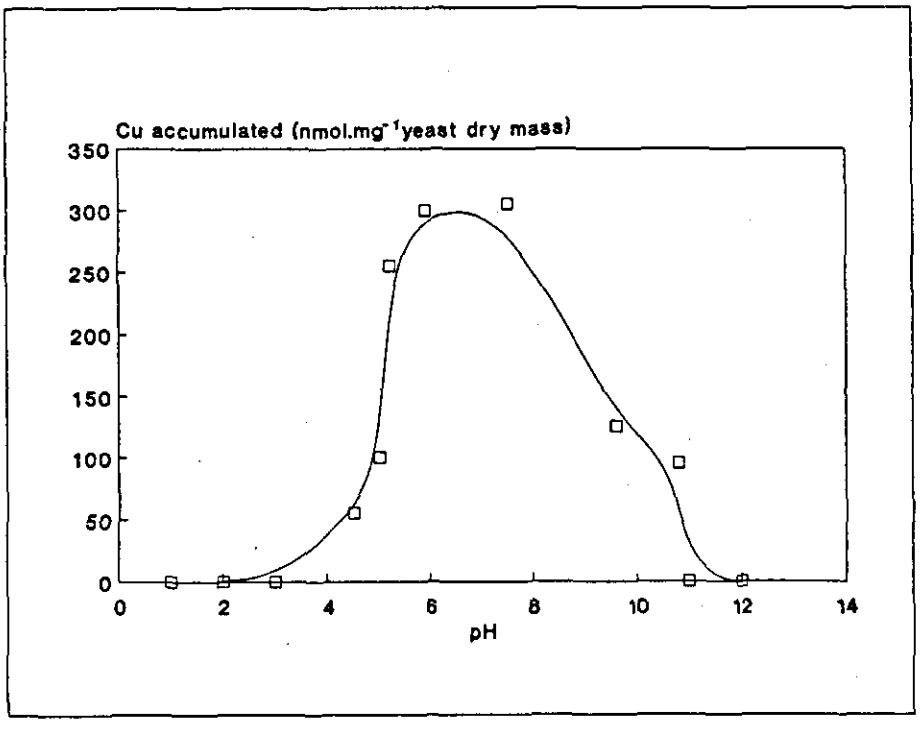


Figure 2.8: Effect of pH on the bioaccumulation of copper by yeast. The pH of the solution was adjusted with HCl or TMAH. Cell concentration was 0.4 $mg.cm^{-3}$ (cell dry mass per volume).

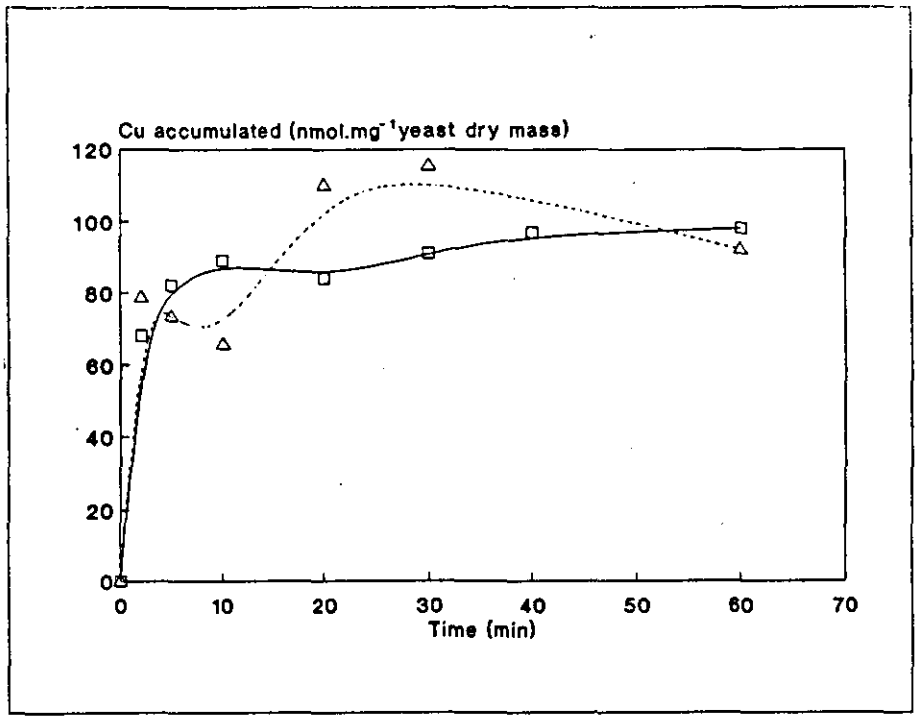


Figure 2.9: Bioaccumulation of copper by yeast in Tris (pH 7.5) or PIPES (pH 6.1) buffers. Cell concentration was 1 mg.cm^{-3} (cell dry mass per volume). Initial copper concentration was 200 μmol.dm^{-3}.

2.3.4 Effect of Temperature on Copper Bioaccumulation:

The effect of temperature on copper bioaccumulation is represented in figure 2.10. Over the range investigated (5° - 40°C), temperature-related effects do not appear to be particularly pronounced. Maximal accumulation occurred at 25° - 30°C.

2.3.5 Effect of Ionic Strength on Copper Bioaccumulation:

From the data in figure 2.11 it appears that *S. cerevisiae* is capable of accumulating considerable quantities of copper cations in the presence of excess monovalent sodium ions with only limited decease in the levels of accumulation at the higher sodium concentrations (approximately a 20% decrease).

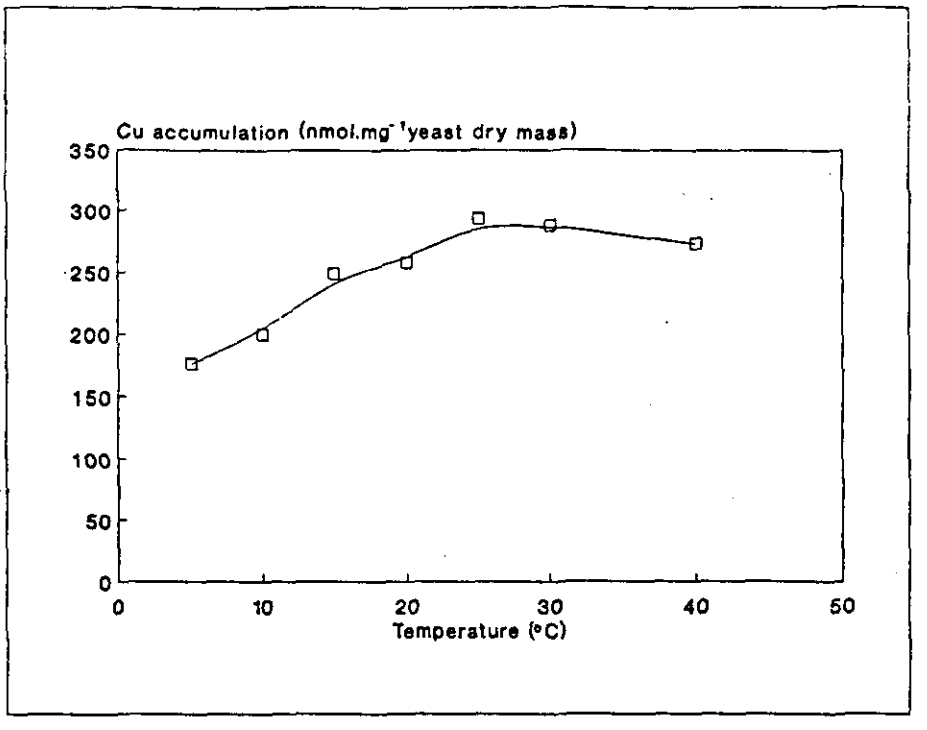


Figure 2.10: Effect of ambient temperature on the bioaccumulation of copper by yeast. Cell concentration was 0.4 mg.cm^{-3} (cell dry mass per volume).

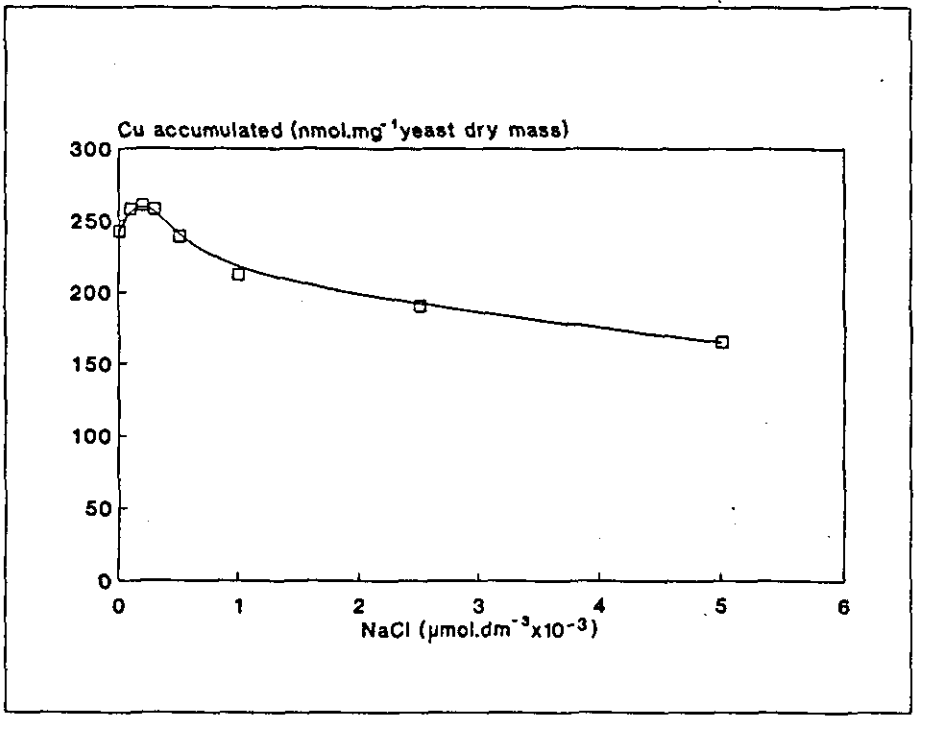


Figure 2.11: Effect of ambient ionic strength on copper bioaccumulation by yeast. Cell concentration was 0.4 mg.cm^{-3} (cell dry mass per volume).

2.3.6 Visualization of the Cell-Copper Interaction using Fluorescent Dye:

The fluorescent dye experiments revealed that the dye would only fluoresce in ultra-violet light in association with the cells if $CuCl_2$ was present in the cell suspension. Solutions containing copper and dye but no cells, or cells and dye but lacking copper, did not fluoresce. It is not possible to determine from these fluorescence photographs (figure 12.12 a, b) whether the dye has been taken up into the cell. The photographs do, however, substantiate the association of the metal with the cells, and the technique could conceivably be modified to yield more visual information about cell-metal interactions. The arrow in figure 11.2 (b) points to a cell which is not fluorescing, suggesting that it has not accumulated copper. This would lend credence to the belief that the cell population is inhomogeneous in its capacity to bioaccumulate metal cations (Passow and Rothstein, 1960).

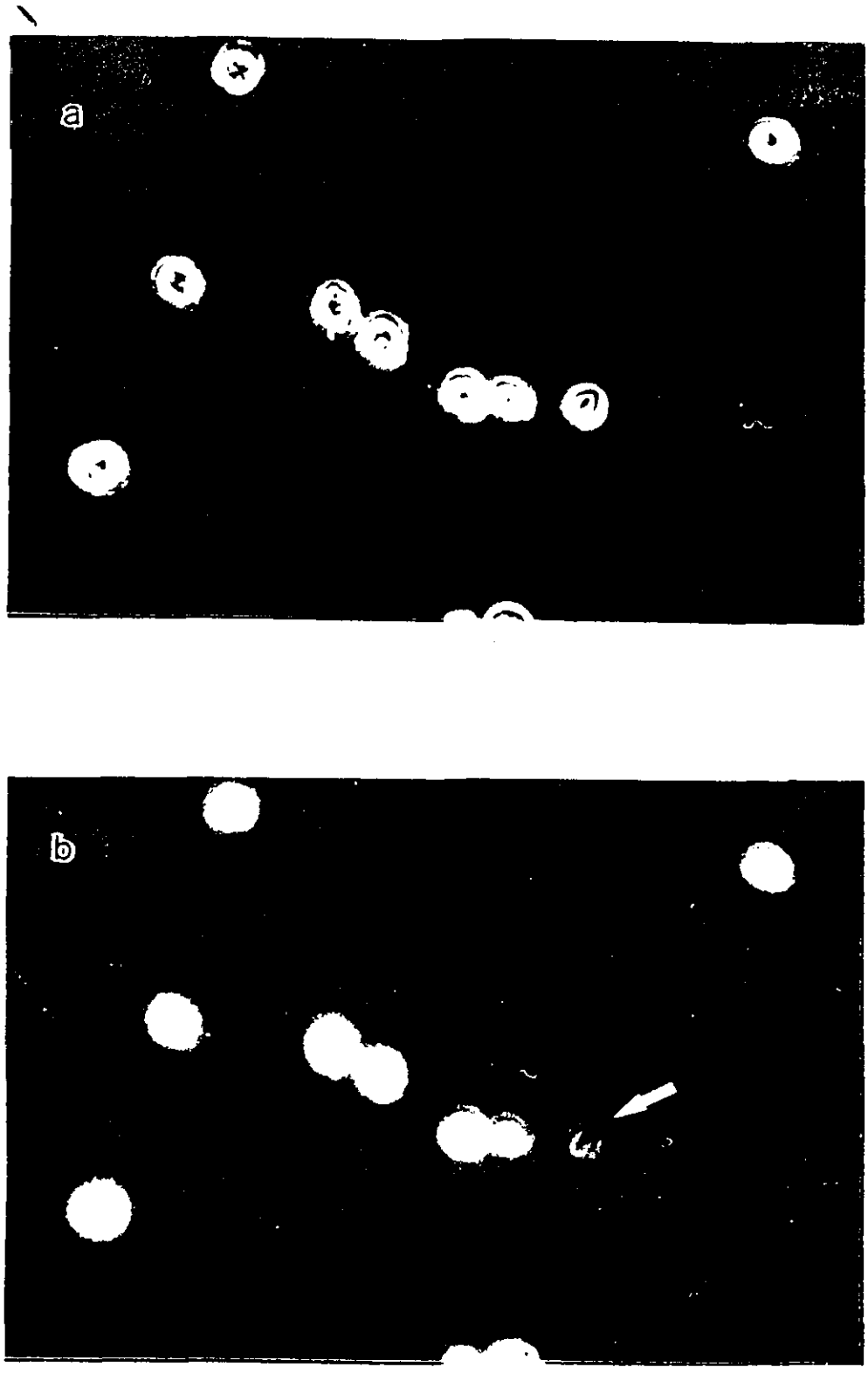


Figure 2.12: Yeast cell fluorescence after copper bioaccumulation in the presence of a fluorescent dye. Photograph (a) was taken under both phase contrast and fluorescent lighting conditions. The same field of view was photographed under fluorescence lighting only in photograph (b).

2.4 DISCUSSION

2.4.1 Bioaccumulation of Heavy Metal Cations:

S. cerevisiae biomass was capable of accumulating Cu, Co, and Cd ions from solutions of the chloride salts of these heavy metals. Similar to the findings of Norris and Kelly (1977, 1979), the process of copper accumulation consisted of two phases, a fast initial phase (within the first 2 min.), and a slower secondary phase. In the present study the second phase did not occur if the ratio of copper to biomass was below a certain limit, suggesting that the two phases are in fact discrete processes. The initial accumulation is probably almost entirely dependent on the biosorption of metal cations to the cell wall. At higher ratios it is likely that a second mechanism, probably metal internalization, become involved (Norris and Kelly, 1977). In many cases initial uptake was followed by a short period during which the total amount of metal accumulated remained constant or decreased slightly before accumulation re-commenced.

In this study 1 mg dry mass of cells accumulated 100 nmol of copper cations and 1 mg dry mass of cells consists of 1.27×10^{7} cells, therefore each cell binds 7.87×10^{-15} mol of Cu^{2+}. This would require a minimum number of binding sites equal to this number. As there are 6×10^{23} Cu^{2+} ions per mol, this suggests that there are approximately 4.7×10^{9} Cu^{2+} binding sites per cell.

The lack of difference in metal accumulation between cell suspensions incubated with or without addition of glucose may indicate that either the cells are not starved or that it was not an energy dependent

process. This is in accordance with other studies (Norris and Kelly, 1977).

Also shown in another study is the fact that addition of substrates need not necessarily lead to enhanced metal accumulation. When sodium acetate or glucose was added to cell suspensions in buffer, the amount of Ni^{2+} retained after hours of incubation were diminished compared to cell suspensions where these substrates were not available (Bordons and Jofre, 1987).

The substrate need not actively stimulate the cell to enhance accumulation of certain metals. For example co-accumulation of ions sometimes occurs with substrate uptake. *Pseudomonas putida* may accumulate germanium in the presence of certain substrates such as catechol. Germanium accumulation by *P. putida* increased with the induction of a transport system to facilitate the internalization of catecol, a carbon source for the bacteria, and corresponded to the linear degradation of the catechol (Chmielowski and Klapcinska, 1986). The germanium ions formed complexes with catechol, and the catechol-germanium complexes were taken up via the inducible catechol transport system. Germanium in the form of non-complexed ions was bio-accumulated to a much smaller degree. This is a form of non-specific transport of metal ions where the ions are complexed with substrates serving as carrier molecules via a transport system specific for these substrates (Chmielowski and Klapcinska, 1986). Amino acids are known to chelate ions and it is possible that ions present as amino acid chelates may be more easily assimilated by microorganisms such as yeast (Jones, 1986).

2.4.2 Relationship of Copper Bioaccumulation to the Ambient Copper Concentration:

Cell cation uptake is partially determined by the ratio of ambient metal concentration to cell biomass. This effect has been noted elsewhere. Uranium accumulation by *S. cerevisiae* was dependent on ambient uranyl ion concentration (Shumate *et al*, 1978), while the uptake of uranium by filamentous bacterium *Streptomyces longwoodensis* was found to be approximately proportional to cell concentration (Friis and Myers-Keith, 1986). The intracellular concentration of manganese was found to be dependent upon the external concentration of the ion solution (Bianchi *et al*, 1981), and there was an almost linear correlation between increasing extracellular Ni^{2+} concentration and accumulation of nickel by a strain of a *Pseudomonas* sp. isolated from nickel-loaded active sludge (Bordons and Jofre, 1987).

Yeasts grown in mercury (II) chloride show a movement of bound mercury from the cell wall to the cytoplasm (Murray and Kidby, 1975). The movement of mercury into the cell is also a function of the external mercury concentration, increasing mercury leads to higher levels in the cytoplasm (Brown and Smith, 1977). This indicates that it is only when most of the metal cation binding sites on the cell wall are occupied that internal accumulation begins. If the cell wall metal binding sites vacated during internalization of the metal can be re-occupied by new external metal then this process would continue until some form of equilibrium was achieved.

2.4.3 Effect of pH on Copper Bioaccumulation:

In the present study it was found that extremes of ambient pH (pH < 5 and > 9) were inhibitory to copper accumulation. Jones and Greenfield (1984) found that the uptake of divalent ions by S. cerevisiae is significantly reduced below pH 5.0. In many cases heavy metal toxicity is reduced at acidic pH values, despite the often increased metal availability, possibly owing to decreased uptake (Gadd, 1990b). A low pH may increase metal mobility, whereas near and above pH neutrality, insoluble oxides, hydroxides and carbonates tend to form (Huang *et al*, 1988). Copper hydroxide formation occurs above pH 5.0 (Norberg and Rydin, 1984). However, the quantity of copper hydroxide formation depends on its association constant compared to the association constants of the cell ligands. Also available anions such as CO_3^{2-}, S^{2-}, PO_4^{3-}, *etc.* can decrease metal availability by precipitation of the corresponding combinations of carbonates, sulphides and phosphates.

The biological availability of a metal is determined by its chemical speciation (*i.e* its charge). Shifts in pH affect metal cation bioaccumulation due to both changes in metal speciation and hydrogen ion competition at the cell surface. Metal binding to organic ligands is inhibited as metal complexes with both organic and inorganic ligands tend to dissociate with decreasing pH of the solution (Schenck *et al*, 1988).

The accumulation of alkali metal cations is reduced at low pH. For instance, as pH is reduced, the net rate of potassium uptake not only decreases but also may even result in loss of intracellular potassium

at pH levels below 2. At very high concentrations of potassium, however, all effects of H^+ are virtually overcome as the potassium can effectively compete with the hydrogen ions (Armstrong and Rothstein, 1964). However, specific active accumulation processes may negate the effects of ion competition. It was found that contrary to expectations, the cellular levels of Mn^{2+}, Fe^{2+} and Ca^{2+} in *S. cerevisiae* increased with decreasing external concentrations of these cations (Jones, 1986). Also the rate and total level of the accumulation of certain metals such as zinc by yeasts (in this case *Candida utilis*) may depend on the stage of the growth cycle which the organism is in (Failla and Weinberg, 1977).

Alteration of pH over a wide range does not measurably alter the average intracellular pH (Rothstein and Hayes, 1956). According to Rothstein and Hayes (1956) this implies that the binding sites must be located peripherally, under the influence of extracellular pH rather than sites exposed to the constancy of intracellular pH. This view does not take into account that a proton gradient across the cell membrane must be present to allow for cation uptake (as will be shown in chapter 7).

The filamentous fungus *Rhizopus arrhizus*, grown on support beads, was used to accumulate cadmium. Accumulation was rapid and was optimal at pH 6 - 9. Cadmium accumulation was, however, reduced by competition with divalent cations and the biomass showed a greater affinity for copper than for cadmium (Lewis and Kiff, 1988). Dead *R. arrhizus* cells are capable of accumulating Cd^{2+}, Zn^{2+} and Ni^{2+} (Roux *et al*, 1991).

2.4.4 Effect of Temperature on Copper Bioaccumulation:

In the present study maximal copper accumulation was in the 25° - 30°C range, although there was little variation in bioaccumulation over the 20° - 40° range. The accumulation of uranium by *S. cerevisiae* has been shown to be greatest at 40°C (Shumate *et al*, 1978). Accumulation processes which depend on cellular metabolism, such as active uptake, would be those that are the most likely to be inhibited by low temperatures, while high temperatures could affect the integrity of the cell membranes and hinder compartmentalization of metal ions, also leading to reduced uptake levels.

2.4.5 Effect of Ionic Strength on Copper Bioaccumulation:

In the present study there was a reduction of approximately a 20% in copper accumulation in the presence of 5 000 $\mu mol.dm^{-3}$ sodium chloride when the initial ambient copper concentration was 200 $\mu mol.dm^{-3}$. This indicates that yeast cells are selective in their uptake of metal cations and that the mechanisms of heavy metal uptake are not overwhelmingly inhibited by elevated ionic strength. This is similar to findings with marine bacteria which have been shown to accumulate high levels of lead from sea water in spite of the relatively high salt levels found in this medium (Thompson and Watling, 1987).

2.5 CONCLUSIONS

Microbial bioaccumulation of metals has many potential applications. Yeast biomass is capable of accumulating heavy metal cations from aqueous solutions. The mechanisms of accumulation and the quantities of copper accumulated depend on the ratio of ambient metal concentration to biomass quantity. Copper accumulation is only slightly affected by the variations in temperature that could be expected in most applicable industrial environments, or by high ambient ionic strength, but extremes of pH are highly detrimental to copper accumulation.

Yeast must be retained, however, if it is not to be leaked into the effluent stream with its cargo of accumulated toxic metals. A particularly cost-effective method of retaining biomass is tangential flow microfiltration; this is the subject of the next chapter.

3. TANGENTIAL FLOW MICROFILTRATION

3.1 INTRODUCTION

Essentially, tangential flow membrane filtration is a technique whereby constituents are separated according to their physical size. Tangential flow membrane filtration allows for efficient solid/liquid separation and has recently begun to replace more traditional methods such as centrifugation because it is less expensive in both capital outlay and running expenses, and is less laborious to operate.

During conventional perpendicular filtration the liquid flows at 90° to the membrane surface and solids in the liquid are retained and consequently deposited on the membrane. The continuing build-up of low porosity material on the membrane results in a layer which continually deepens and consequently reduces the flux of liquid across the membrane as it does so. Moreover, biological material is often gelatinous and is therefore prone to compression under the pressures used during filtration, the result of which is to reduce the porosity to an even greater extent. The alternative is to use tangential flow filtration where the deposited layer is continually washed away as the liquid passes over the membrane in a flow parallel to the membrane surface (figure 3.1). Increasing tangential velocity produces higher shear forces with proportionally reduced deposit thickness (Bindoff, 1988). An equilibrium between solid deposition and removal develops, ensuring that the layer of deposited solids remains constant.

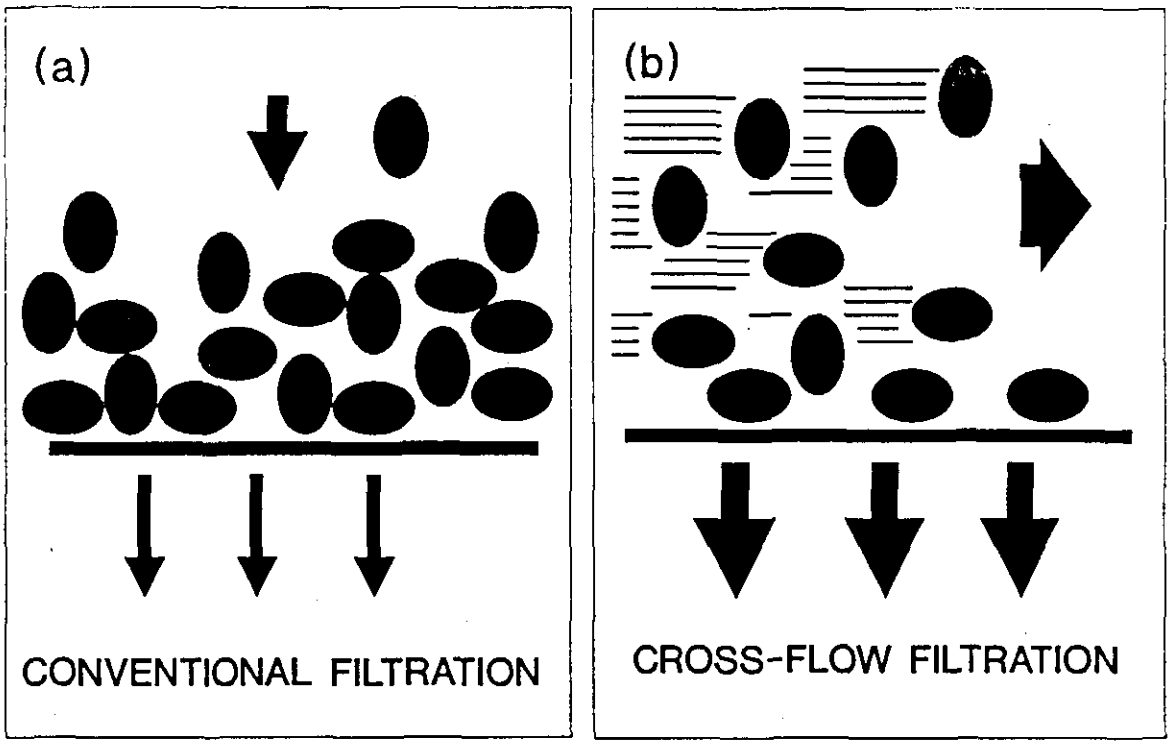


Figure 3.1: Schematic representation of conventional (a) and tangential flow (b) filtration processes.

There are three types of tangential flow membranes, classified according to the pore size of the membrane. Reverse osmosis is used for desalination and operates at very high pressures of up to 6 000 kPa, as it must overcome osmotic pressure. Ultrafiltration (UF) uses pressures an order of magnitude less than reverse osmosis and is used to retain macromolecules and colloids. Cross-flow micro-filtration (CFMF) operates at even lower pressures and is used to retain particulates, microorganisms and certain colloids. The size of the pores in the membrane is smallest for reverse osmosis systems and largest for cross-flow microfiltration systems. The installation and operation costs increase with pressure applied to the system.

Any of the above membrane systems could be used for cell harvesting. However, in practice microfiltration systems are preferred because they are capable of the highest fluxes (volume of permeate per time and per unit area of membrane) and require the lowest operating pressure. This obviously results in the lowest possible operating costs (Le and Atkinson, 1985). Most UF systems work at less than 700 kPa, and hollow fibre CFMF systems at less than 300 kPa. Reverse osmosis membranes, although ideal for removal of metal ions from solution, are prohibitively expensive to install and operate for routine wastewater purification.

There is already considerable expertise in cell separations and processing using tangential-flow membrane filtration technology (Gabler, 1985; Tutunjan, 1985). Tangential flow systems have been demonstrated to be useful in harvesting cells and cell debris (Le and Atkinson, 1985). *Saccharomyces cerevisiae* cultures have previously been maintained using cross-flow membrane filtration units to retain the biomass and yet allow exchange of liquids without damage to the yeast (Uribelarrea *et al*, 1990). *S. cerevisiae* has also been harvested by CFMF during cider fermentations (Scott, 1988). Moreover, CFMF has been utilized in wastewater management systems, such as retention of biomass in a two phase anaerobic digestor (Anderson *et al*, 1986).

A new innovation in membrane technology, hollow fibre bioreactors, may be used to retain biomass at high concentrations and allow solutions such as wastewaters to pass through them (Dall-Bauman *et al*, 1990). Here fixed-films of cells are maintained in low shear conditions at higher cell densities than are practical with suspended cell reactors.

A unique benefit of hollow fibre systems is that they may be back washed (i.e. the flow of the permeate is reversed) to dislodge particles which have clogged the pores (Le and Atkinson, 1985). They also allow for large membrane surface areas in relatively small volumes. Hollow fibre cross-flow microfiltration also conveniently allows for cell washing processes (Le and Atkinson, 1985), which could be necessary for metal recovery from the cells using chemical elutants.

However, even with back washing, membrane fouling may still present problems. Protein deposition on the walls of the pores can reduce pore volumes and hence decrease membrane permeation rates (Bowen and Gan, 1991), while adhesion of cells or cell debris can cause bio-fouling of the surface of the membrane, again reducing flux rates (Defrise and Gekas, 1988). By varying the hydrophobicity/ hydrophilicity of the membrane by use of alternative materials for polymer matrix formation, it is possible to limit the level of membrane fouling by proteins and other biological polymers (Capannelli *et al*, 1990).

Flux rate:

The permeation rate is the rate of passage of liquid through the membrane. This value is unique to each membrane. The flux rate is the rate of permeation of liquid through the membrane per unit surface area and hence this value is applicable to all membranes of the same type, of any size, provided the membrane surface area and operating conditions are known. The factor which determines the flux rate is pressure. The transmembrane pressure is the difference in pressure on

the feed side of the membrane (P_i) compared to that on the permeate side of the membrane (P_o), the pressure gradient being maximal at the inlet and at its minimum at the outlet. The average transmembrane pressure (PTM) would then be:

$$\Delta PTM = (P_i + P_o)/2 - P_f$$

Where P_f is the permeate pressure, which in most examples is small enough to be ignored.

The flow across the membrane sets up a pressure gradient in itself where the tangential flow pressure gradient (ΔP) is:

$$\Delta P = P_i - P_o$$

The tangential flow pressure is related to the transmembrane pressure at very low permeate pressures by the equation:

$$\Delta PTM = P_i - (\Delta P/2)$$

Flux rate is dependent on the operational temperature, with increased temperatures allowing for faster flux rates (Scott, 1988). Flux rate may even decrease during filtration of pure water, a phenomena that has been attributed to the compaction of the microporus film (Lopez-Leiva and Gekas, 1986).

3.2 ULTRAFILTRATION SYSTEMS

3.2.1 MATERIALS AND METHODS

The use of ultrafiltration was investigated to determine its potential for harvesting yeast cells after bioaccumulation. The equipment used is shown schematically in figure 3.2.

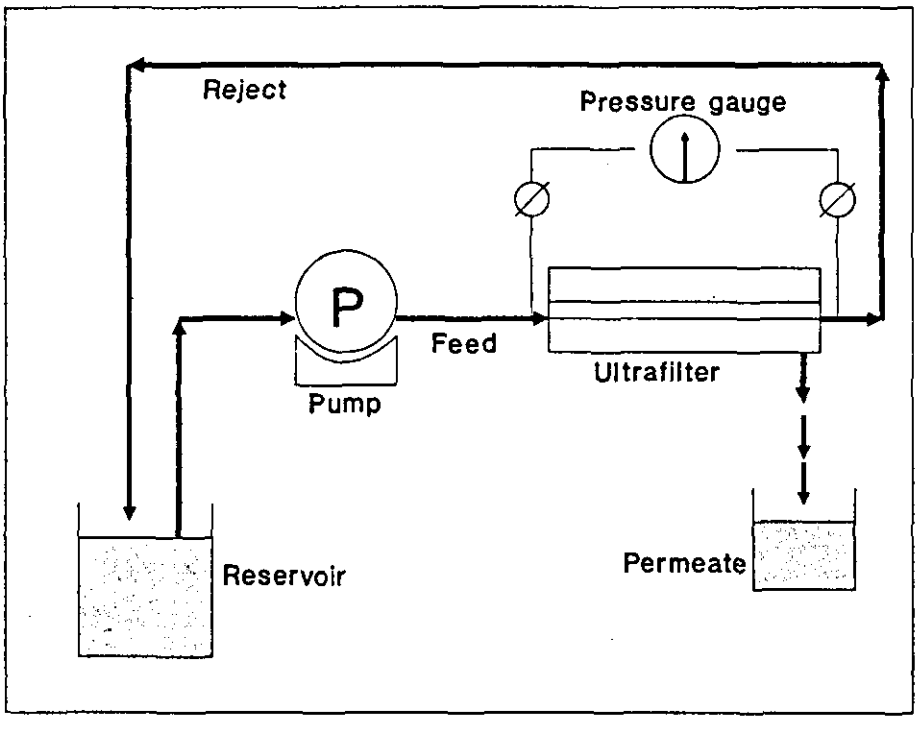


Figure 3.2: Cross-flow ultrafiltration system. Yeast cell suspensions are pumped under pressure into the lumen of the hollow fibres within the hollow fibre cartridge, and the resultant permeate drains to the exterior where it is collected.

Experimental conditions:
Temperature: 20°C
Inlet pressure: 115 kPa; outlet pressure: 30 kPa; pressure difference: 85 kPa. Calculated transmembrane pressure: 72.5 kPa.
Reject flow rate: 250 $dm^3.hr^{-1}$
Membrane area: 9.4 $x10^{-3}$ m^2
Membrane type: Polysulphone ultrafiltration hollow fibre membrane.
Molecular mass cut-off: 20 - 80 kDaltons. Cut-off diameter < 0.1 μm.
Pump type: positive displacement Mono pump.

Determination of flux rate during harvesting of *S. cerevisiae*:

Yeast cells (10 g wet mass) were suspended in 3 dm^3 ultra-pure water and harvested according to the experimental conditions stated above.

Determination of initial flux rates:

One cubic decimeter of 5 $mmol.dm^{-3}$ HEPES buffer pH 7.2 containing 200 $\mu mol.dm^{-3}$ $CuCl_2$ and 3.3 g wet mass of yeast cells was harvested as stated above while the initial flux rate was determined by measuring the permeate flow rate and dividing it by the area of the membrane. No obvious cell lysis was noted.

3.2.2 RESULTS AND DISCUSSION

The polysulphone ultrafiltration system was demonstrated to be capable of harvesting *S. cerevisiae* cells without causing excessive cell damage and with little decrease in flux compared to the initial flux rate (figure 3.3). Moreover, the initial flux rate of native cells and cells which had accumulated copper were similar (results not shown).

Attempts to measure Cu levels in the filtrate were unsuccessful. Little or no copper was found in the filtrate even if no cells were present in the copper solution. This may have been caused by precipitation of copper ions on the metal components of the system (i.e. the pump and the pressure gauge). The overall efficiency of metal accumulation of this type of system is therefore difficult to determine with this particular apparatus.

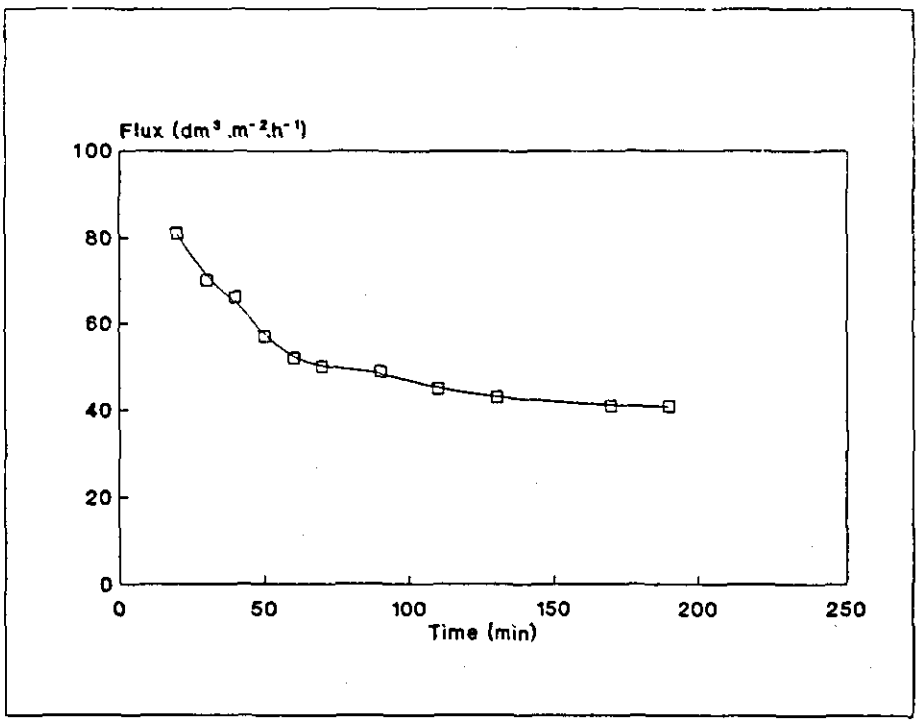


Figure 3.3: The flux rate of yeast cell ultrafiltration using a polysulphone hollow fibre cartridge.

3.3 MICROFILTRATION SYSTEMS

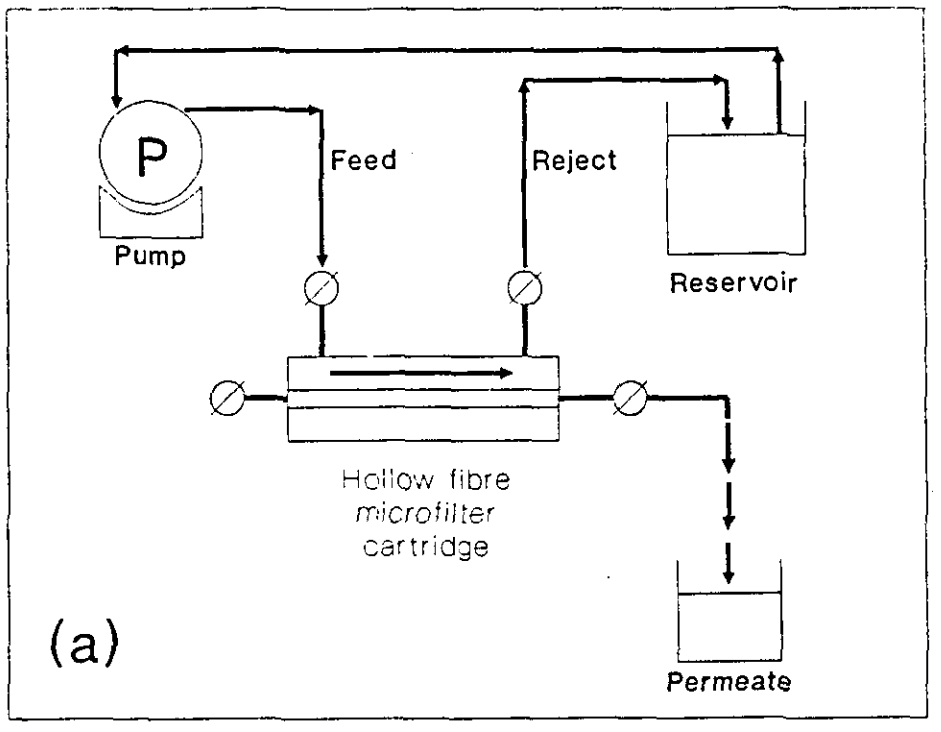
An alternative apparatus was assembled using a microfiltration hollow fibre membrane system. This system functioned under much lower pressures than ultrafiltration systems, allowing the use of peristaltic pumps instead of displacement pumps. This apparatus did not remove copper from solution (as was the case with the ultrafiltration apparatus used above). This system was used for harvesting of the yeast biomass after bioaccumulation of copper. Alternatively, the biomass was initially harvested onto the membrane and bioaccumulation occurred when buffered copper solutions were filtered through the biomass-packed membranes.

3.3.1 MATERIALS AND METHODS

A polypropylene microfiltration hollow fibre membrane was used. Pore size was 0.1 μm, P_i was maintained below 10 bar. The microfiltration cartridge was supplied by Dr E. P. Jacobs of the Institute for Polymer Science, Stellenbosch University, RSA. The equipment set-up is depicted in figure 3.4. Unlike the ultrafiltration experiments the yeast cell suspensions were circulated around the exterior of the hollow fibres and the permeate passed into the lumen.

(a) Serial microfiltration for bioaccumulation of copper.

S. cerevisiae cells (1.4 g wet mass) were suspended in a 1 dm^3 solution of 200 $\mu mol.dm^{-3}$ $CuCl_2$ buffered with 5 $mmol.dm^{-3}$ PIPES, pH 6.5. The temperature of the reaction suspension in the reservoir was maintained by a thermostatic bath set at 30°C. The cells were harvested after 10 minutes of bioaccumulation time by CFMF. The system was run as a dead-end (perpendicular) membrane system with no tangential flow, by closing the reject valve. Constant addition of further copper stock solution (200 $\mu mol.dm^{-3}$ $CuCl_2$) maintained the volume in the influent reservoir at its initial level while permeate was collected in separate 1 dm^3 volumes. After biomass saturation with copper, cells were backwashed out and replaced by fresh yeast cells prior to the next bioaccumulation cycle, where the effluent from the first cycle was used as the influent for the second cycle. Separate 1 dm^3 volumes of permeate from the first cycle were processed in the second cycle in the same sequence as they were collected. The same procedure was repeated to give a third cycle using second cycle effluent as influent.



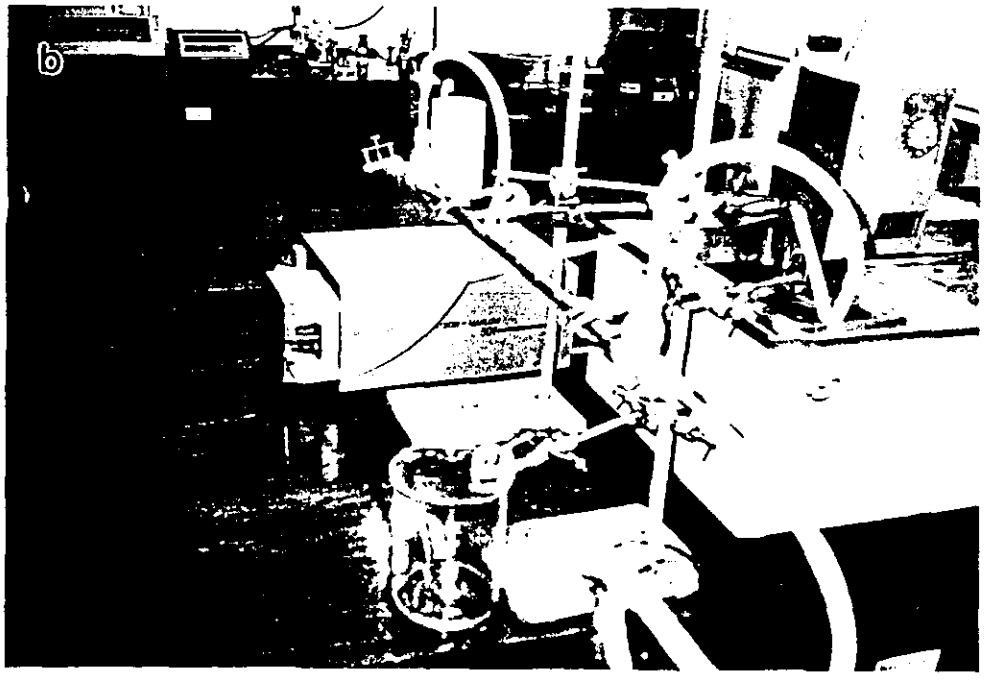


Figure 3.4: Microfiltration equipment set-up, both schematically (a) and a photograph of the actual cross-flow microfiltration system used (b). There were 7 hollow fibres within the cartridge with a total surface area of 0.013 m^2.

(b) Pre-packing of microfiltration filter cartridges with yeast:

As opposed to using reactor vessels for bioaccumulation followed by harvesting, bioaccumulation by biomass packed onto or into the hollow fibre would have many advantages.

Ten times the mass of cells used in experiment (a) (i.e. 14 g of yeast) was suspended in buffered solution and pumped into the filter cartridge. Immediately buffered $CuCl_2$ stock solution (200 $\mu mol.dm^{-3}$) was added to the reservoir in such a manner as to maintain a constant volume in the reservoir during the period of the experiment. This was compared to a parallel experiment where a similar mass of cells in suspension was packed into the filter cartridge by harvesting prior to addition of metal solution to the reservoir. This was achieved by diverting the permeate outlet back into the reservoir until packing was completed. The packing of the filters with cells could be followed by measuring the absorbance (spectrophotometrically at 540 nm) of the solution in the reservoir, to which all yeast biomass not deposited on the filter membrane must return.

(c) Bioaccumulation of metal cations from contaminated tapwater:

Potential applications of bioaccumulation include the restoration of heavy metal contaminated waters. To simulate this type of application, a CFMF filter, packed with yeast biomass, was used to filter heavy metal-doped tapwaters with the aim of accumulating these metals and thereby restoring the water.

The capacity a microfiltration cartridge packed with 14 g wet mass of yeast cells to bioaccumulate various metals (Cu^{2+}, Cd^{2+}, Pb^{2+}, and

Cr^{3+}) from metal-doped tapwater was determined. In each example the influent metal concentrations were 200 $\mu mol.dm^{-3}$. A single metal was added to tapwater during each experiment.

(d) The effect of metal ions on the flux rate of yeast loaded microfiltration units:

One of the most important parameters of membrane filtration performance is the flux of solution across across the membrane as this determines the physical attributes of a CFMF system required. The flux rate of tapwater across a CFMF filter packed with 14 g wet mass of yeast cells was determined.

3.3.2 RESULTS AND DISCUSSION

The use of sequential heavy metal accumulation by biomass was demonstrated to be effective (figure 3.5), but the intracellular cation release by the cells accumulates downstream in the process (figures 3.6, 3.7 and 3.8) and could result in cation competition for binding sites in later cycles. The increase in copper in the first samples of series 2 and 3 (figure 3.5) indicates that there are probably still copper-saturated cells associated with the membrane that were not removed by backwashing, and these may be releasing copper back into the solution as a new equilibrium occurs. This is an experimental artefact caused by the reuse of the same membrane cartridge for all three cycles and would not occur where an actual series of cartridges were used.

The shift in equilibrium concentration of copper produced by the continual addition of fresh metal solution allows the cells to accumulate more metal than would be possible in batch cell conditions in which there is to the constant ambient metal concentration. In this particular experiment complete removal of the metal from solution did not occur, probably owing to lack of sufficient theoretical equilibrium plates. The saturation of the cells with copper in cycle 1 is indicated by a steady increase in effluent copper (figure 3.5).

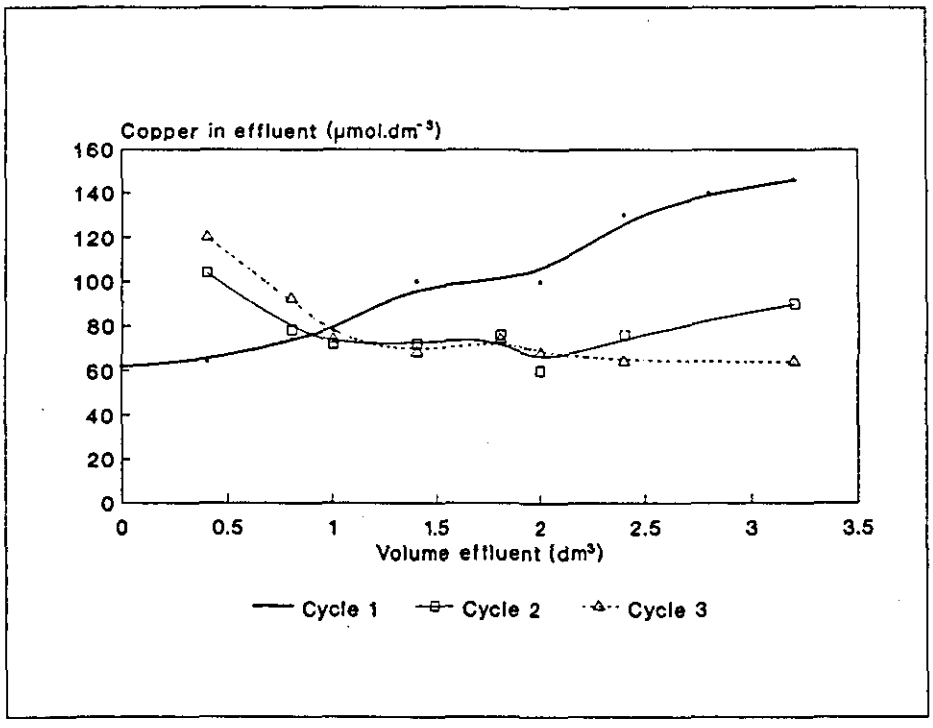


Figure 3.5: Copper accumulation by application of serial cross-flow microfiltration. Reject: 0 $dm^3.h^{-1}$; feed: 0.2 $dm^3.min^{-1}$. Flux rate: 9.2 x 10^2 $dm^3.m^{-2}.hr^{-1}$.

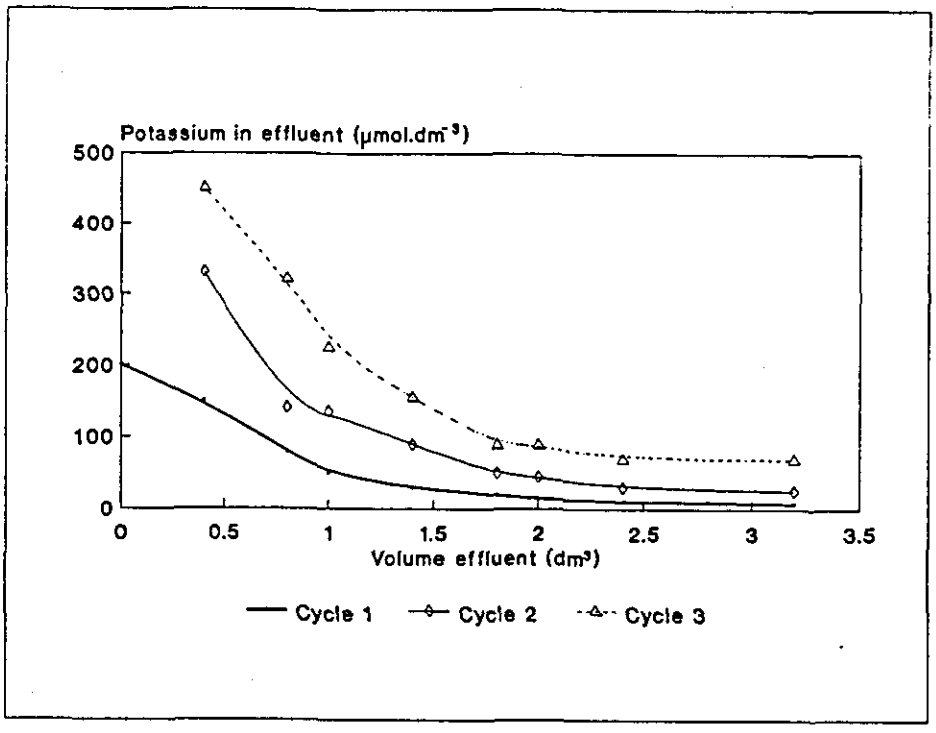


Figure 3.6: Potassium release during copper accumulation by application of serial cross-flow microfiltration.

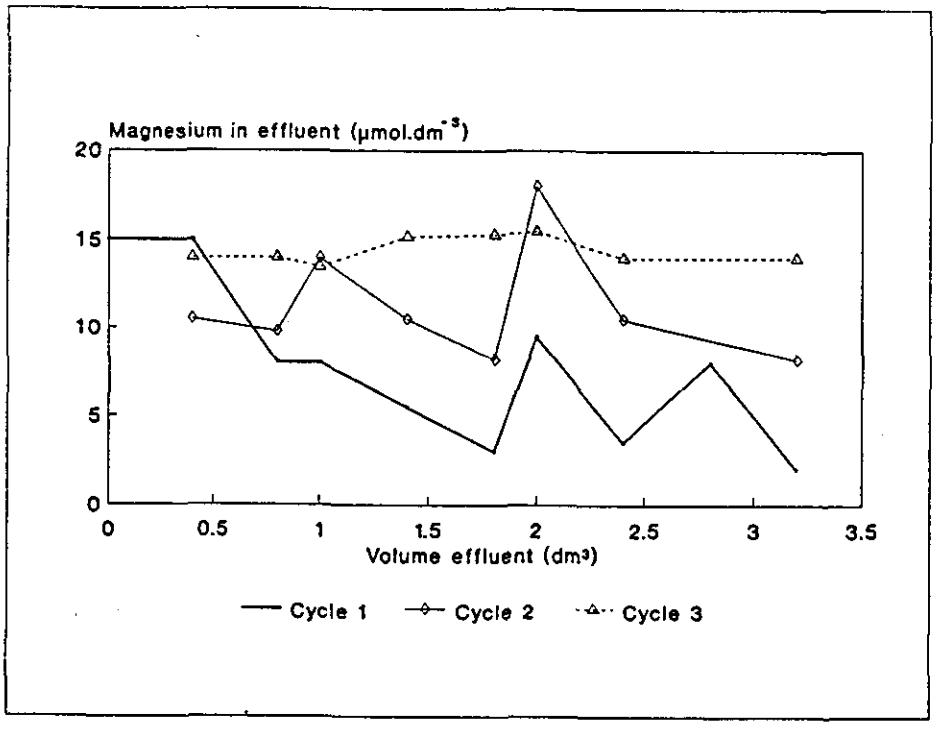


Figure 3.7: Magnesium release during copper accumulation by application of serial cross-flow microfiltration.

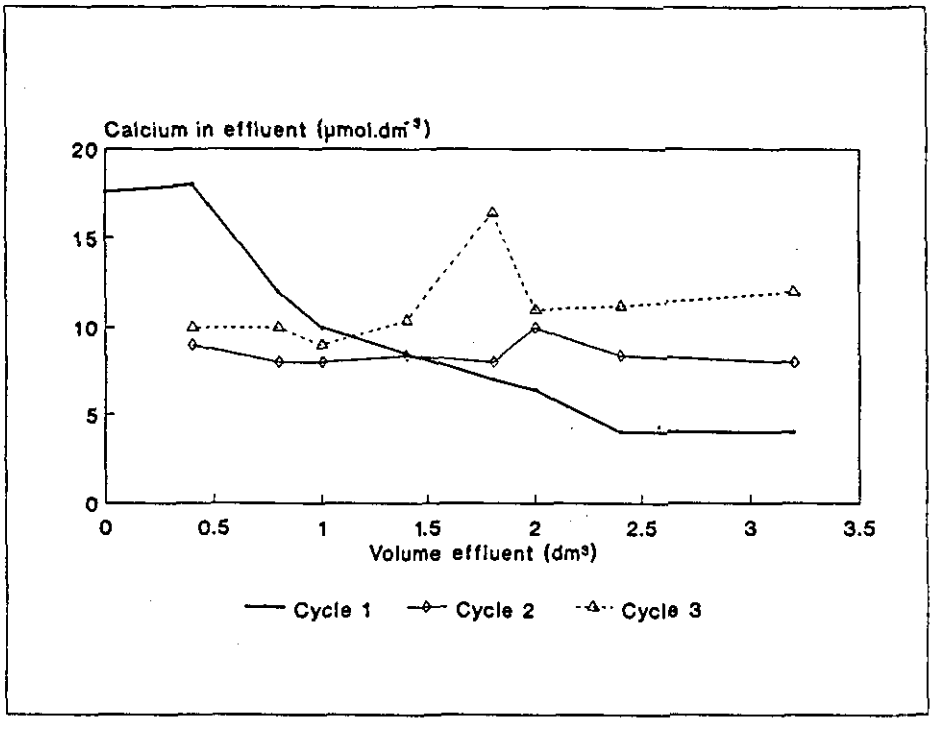


Figure 3.8: Calcium release during copper accumulation by application of serial cross-flow microfiltration.

Harvesting of yeast biomass during copper accumulation (figure 3.9) and an alternative process of pre-packing of microfiltration filter cartridges with yeast prior to accumulation of copper (figure 3.10) were compared (figure 3.11). In each case substantial quantities of copper were accumulated.

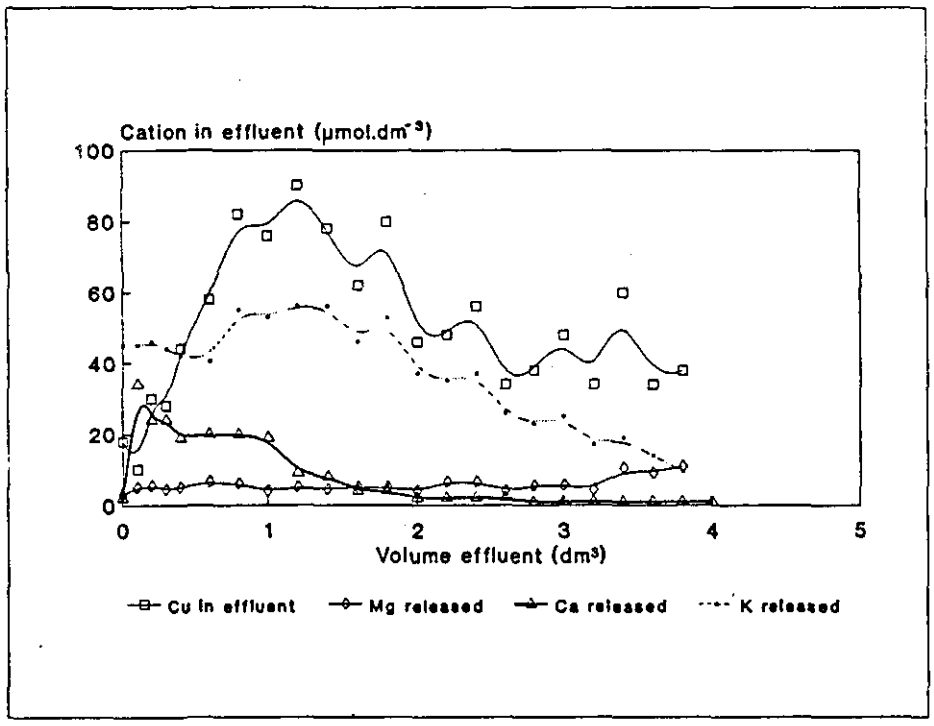


Figure 3.9: Harvesting of yeast by CFMF during bioaccumulation of copper. Temperature: 30°C, flux rate: 9.2×10^2 $dm^3.m^{-2}.hr^{-1}$.

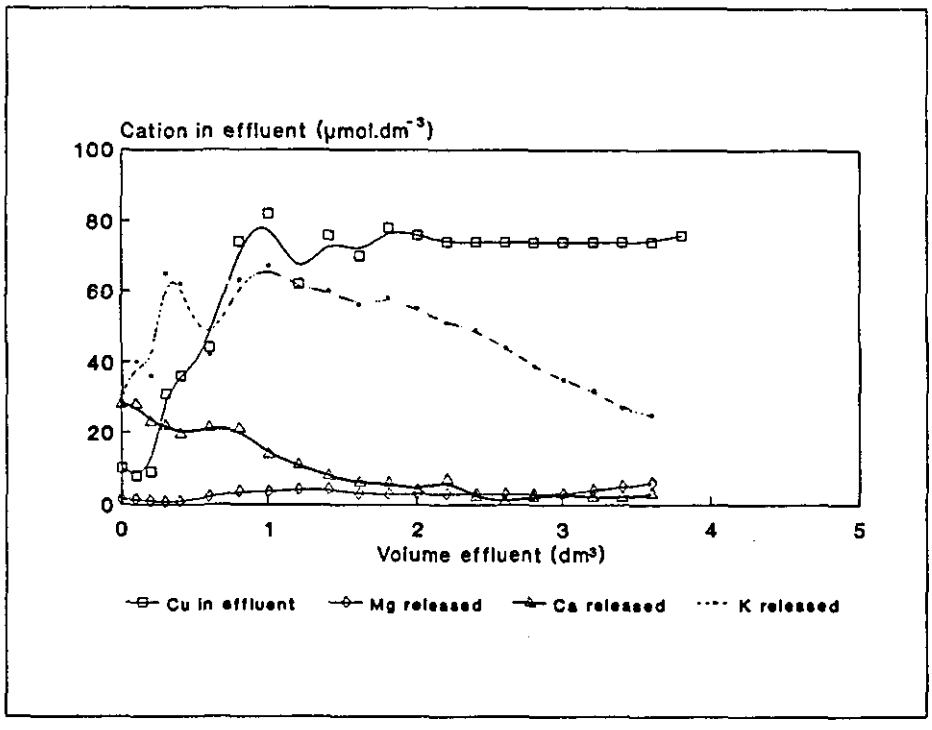


Figure 3.10: Sequential packing of the hollow fibre cartridge with yeast, followed by bioaccumulation. Temperature: 30°C, flux rate: 12.5×10^2 $dm^3.m^2.hr^{-1}$.

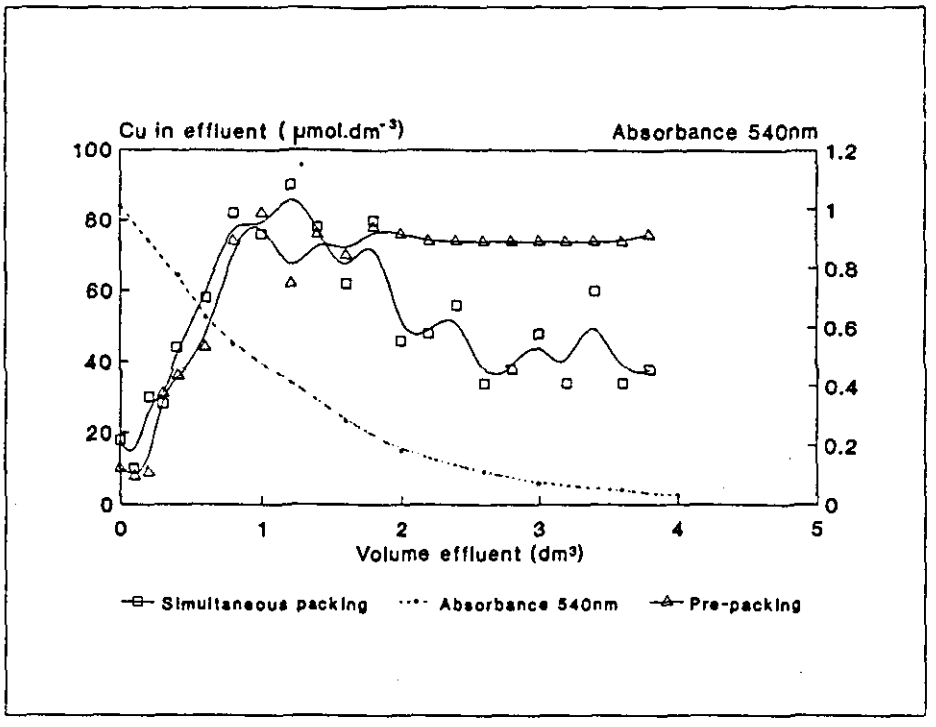


Figure 3.11: Comparison of copper bioaccumulation when either pre-packing the CFMF filter with yeast biomass prior to accumulation or simultaneous accumulation and harvesting. Yeast cell packing onto the CFMF filters during simultaneous harvesting/accumulation is represented by the decreasing absorbance (at 540 nm) of the solution in the reservoir.

The hollow fibre unit that was packed with yeast during simultaneous yeast harvesting and copper accumulation allowed for greater copper removal from solution than did the alternative unit. This was probably due to the slower permeate flow rate, allowing for a longer period of accumulation. The latter system provided a more constant level of accumulation than the former. It was also apparent that the accumulation by the former system improved once the bulk of the biomass had packed onto the membrane, suggesting that passage of copper solution through layers of biomass facilitates greater quantities of copper accumulation than in the case of cell suspensions.

The method of adding stock buffered copper solution to the reservoir means that initally the influent copper concentration is low and only equates to the concentration of the stock solution after approximately 1 dm^3 of influent has been processed; this can be seen in figure 3.11 by the initial low copper concentration in the effluent.

Results of metal accumulation from heavy metal-doped tapwater indicate that this method can be extremely effective in metal removal from heavy metal-contaminated tapwater (figure 3.12). The metals Cr^{3+}, Cu^{2+}, and Pb^{2+} were accumulated in large quantities, while Cd^{2+} was accumulated to a much lesser extent.

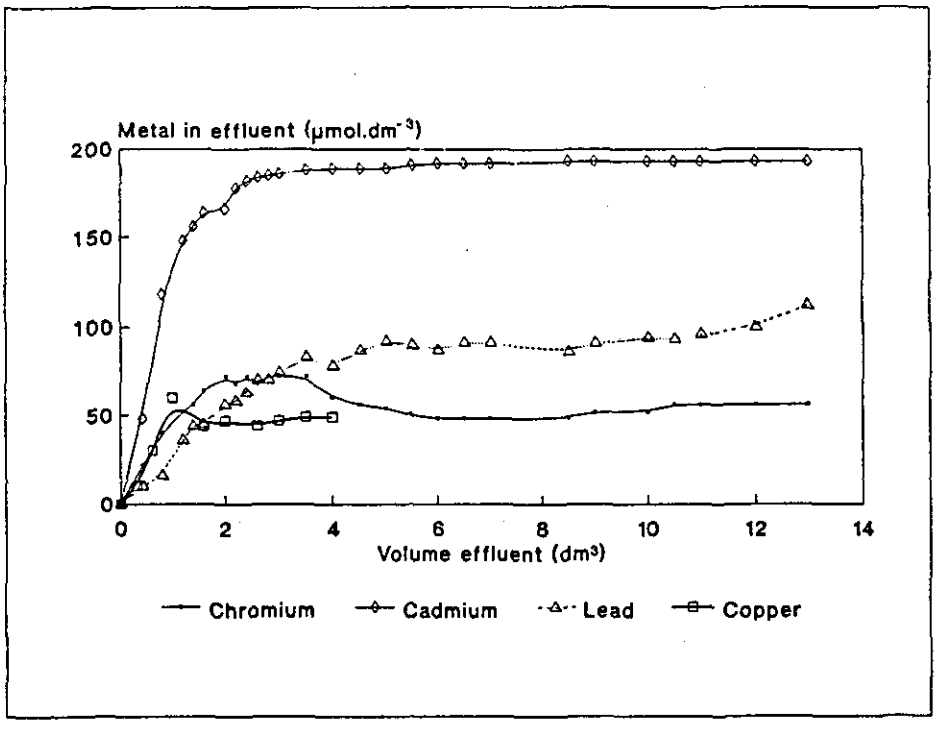


Figure 3.12: Bioaccumulation of metal cations from domestic tapwater artificially contaminated by metal chloride salts. Cu^{2+} accumulation was only monitored for the first 4 dm^3.

The system was also capable of haze removal from lead contaminated tapwater (figure 3.13), possibly reflecting the retention of colloids by the system.

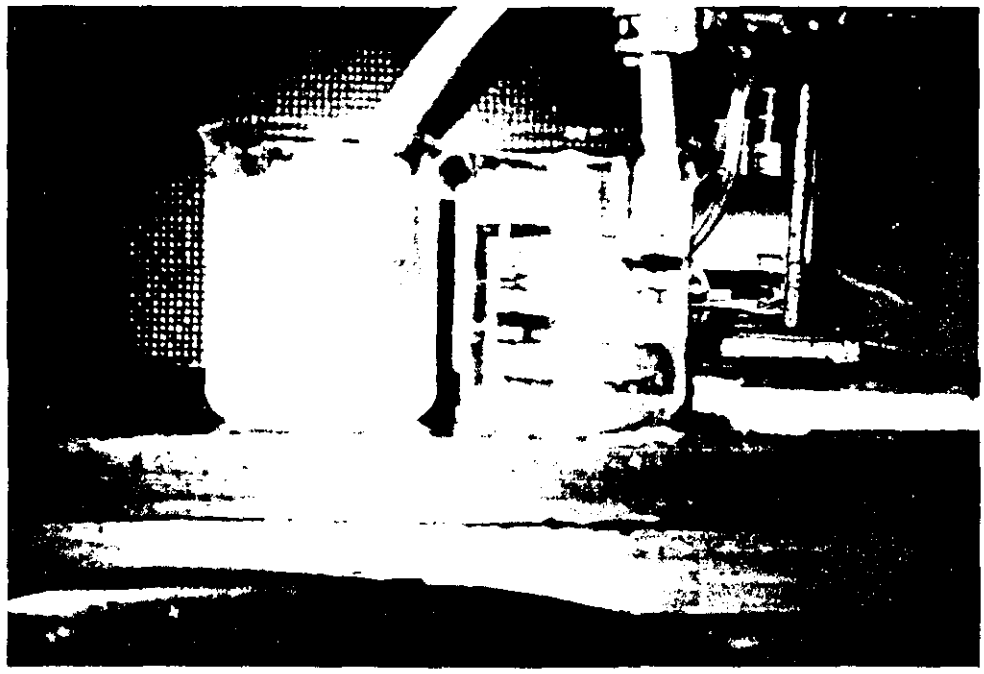


Figure 3.13: Haze removal by the yeast cell coated cross-flow microfiltration unit. The haze observed in the influent solution (left) is removed by the CFMF based bioaccumulation system to yield a clear effluent solution (right).

The variation of flux rate with addition of tapwater to a cartridge packed with yeast cells was determined (figure 3.14). The flux rate increased with passage of permeate across the membrane.

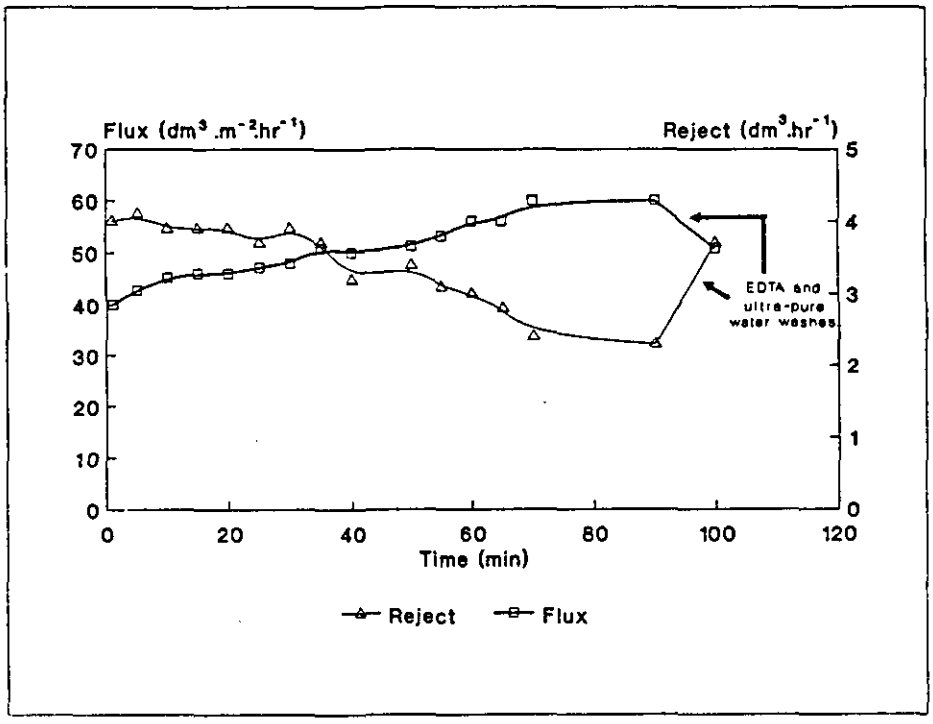


Figure 3.14: The flux rate of hollow fibre cross-flow microfiltration during yeast biomass harvesting as a function of time, and the effect of addition of the chelating agent EDTA.

Unrelated experiments being conducted at the same time on metal binding by yeast cell walls retained on perpendicular flow Millipore filters showed that addition of low concentrations of divalent ions, or even more so with low levels of monovalent ions, resulted in substantial back-pressure compared to when high levels of divalent cations were used. Addition of divalent cations may therefore modify the cell layer, possibly by aggregation of cells such as that seen during flocculation, resulting in channeling of the influent stream, which in turn would allow for greater fluxes. Tapwater is loaded with

cations in the parts per million range and would therefore constitute a source of divalent cations. To detemine if the increase in flux rate was due to the action of metal cations the cartridge was washed with a solution containing a cation chelator (EDTA, 0.4 dm^3 of 1 $g.dm^{-3}$) followed by a wash with ultra-pure water. This washing with a chelating agent demonstrated that the flux rate could be returned to the level measured at the end of the cell packing process. This effect could possibly be explained as the result of the dis-aggregation of the flocculated yeast caused by the chelation of the flocculating agent (multivalent ions) by EDTA. Cation-induced flocculation on the membrane would result in improved flux rates but with consequent reduced contact time and contact surface, and a concomitant decrease in metal accumulation.

Biofilms:

The information available on the formation of biofilms by microorganisms could be useful when studying the behaviour of yeast films on CFMF membranes during metal bioaccumulation. In biofilms, cells are attached to solid surfaces as multicellular films and are often surrounded by an extracellular polymer coat referred to as a glycocalyx, which is extruded by the microorganisms (Blenkinsopp and Costerton, 1991). Biofilms behave biochemically more like multicellular tissues than individual free cells. With the development of heterogeneity within the biofilm, micro-gradients of metabolites, such as oxygen and pH, are produced. The transfer of small molecules in a biofilm is dependent on a number of kinetic parameters such as diffusion rates within the biofilm (Lewandowski *et al*, 1991). Accumulation of ions by biofilms may therefore differ from that of free cells.

The exopolysaccharide components of the glycocalyx have a high affinity for metallic cations, a function that has already been put to good use in certain wastewater treatment plants (Blenkinsopp and Costerton, 1991) as the anionic polymers may inhibit the entrance of cationic molecules into the biofilm by acting as a molecular sieve and an ionic exchange matrix (Lewandowski *et al*, 1991; Lock *et al*, cited by Blenkinsopp and Costerton, 1991). Development of the glycocalyx, however, requires time, and it could easily be removed by harsh treatment such as exposure to high sheer conditions during tangential filtration. There is a dearth of information on the formation of biofilms and glycocaylexes by *S. cerevisiae*.

3.4 CONCLUSIONS

The use of cross-flow microfiltration units in bioaccumulation processes appears promising. The use of serial batteries of CFMF-based yeast bioaccumulators could reduce the concentration of toxic heavy metals in wastewaters significantly. It would also ensure that all particulate matter and harmful microbes were simultaneously removed. As was seen in these experiments the system is also capable of removing haze from metal salt-doped tapwater. Moreover, microfiltration has the potential to remove both particulate metal and metal ions bound to large organic molecules.

In the initial cycle of Cu^{2+} bioaccumulation by yeast being harvested on a microfiltration membrane (experiment 3.3.1(a)) the biomass accumulated 643 $nmol.mg^{-1}$ Cu^{2+} per dry mass with a flux rate of

92 $dm^3.m^{-2}.hr^{-1}$. Using 100 m of this type of cartridge it would be possible to process 2 000 dm^3 of wastewater per hour and remove 0.21 moles (13 g) of Cu^{2+} per Kg (wet mass) of yeast when the influent Cu^{2+} concentration is 200 $\mu mol.dm^{-3}$.

The full potential of CFMF technology in bioaccumulation is not obvious. The application of CFMF is relatively recent and it is often impossible to predict how a system will behave from first principles (Cooney, 1990). The choice of membrane type is important, however, and careful selection of membrane type and system can greatly affect the success of the system (Belfort, 1989). The membranes used for downstream processing should be negatively charged to minimalize biomass adsorption onto the membrane (Defrise and Gekas, 1988), and thereby reduce fouling of the membrane, while negative charges on the membrane may bind metals as a side effect. Further experimentation and experience with CFMF may allow for improvement of the bioaccumulation process in ways that cannot presently be forseen.

Tangential flow technology is applicable to bioaccumulation as it may be used to retain biomass prior to or during the bioaccumulation process. There is another way to achieve these two functions, and that is by the process of immobilization. Similar to, but to a greater extent than pre-packing of cross-flow filters with biomass, the biomass is fixed in place by immobilization. Immobilization has the advantage, however, of allowing a more varied range of configurations of biomass and bioaccumulation systems. Bioaccumulation by immobilized biomass is the topic of the next chapter.

4. IMMOBILIZATION OF YEAST FOR BIOACCUMULATION OF METAL CATIONS

4.1 INTRODUCTION

Cell immobilization is a technique that has developed to allow for high cell concentrations in bioreactors with reduced cell loss during processing. The typical methods of immobilization involve entrappment of the cells in a three dimensional matrix or alternatively binding the cells to inert substrates and/or each other via chemical cross-links. A fortunate side effect of immobilization is that it often improves the stability of the cells and thereby ensures a longer useful existence (Klein and Vorlop, 1985; Nakajima and Sakaguchi, 1986). All of these properties are potentially useful in heavy metal bioaccumulation processes. The process of immobilization is, however, quite complex and expensive, which may be prohibitive to its industrial application, especially in bioaccumulation of toxic metals which is not necessarily a profitable endeavour.

Immobilization has been used with some success in the field of bioaccumulation of metals. Immobilization of the fungus *Rhizopus arrhizus* has been particularly successful (Ileri *et al*, 1990; Lewis and Kiff, 1983; Tsezos, 1984, 1990). For instance *R. arrhizus* was utilized by Tsezos *et al* (1989) to recover uranium from ore bioleach.

Yeasts, too, may be immobilized in a variety of materials such as carrageenan (del Rosario and Pamatong, 1985), or alginate (Flink and Johansen, 1985), or to gelatin (Doran and Baily, 1985), or sintered

glass (Bisping and Rehm, 1986). Yeasts are found naturally immobilized in trickling filters of wastewater works (Dart and Stretton, 1980).

Polyacrylamide immobilization has been determined to be superior to calcium alginate, gluteraldehyde, agar or cellulose acetate immobilization when used for uranium adsorption by biomass, and furthermore this immobilization process only slightly decreases metal adsorption properties of the biomass (Nakajima *et al*, 1982). Polyacrylamide immobilization has an advantage that is not prone to damage by cation replacement or chelation as calcium alginate systems are - an important attribute when accumulating metal cations. Moreover, alginate systems are unstable at high pH (Klein and Vorlop, 1985). Additionally, polyacrylamide has been found to be capable of specifically accumulating mercury cations (Hg^{2+}) even in the absence of biomass (Darnall *et al*, 1986).

The yeast used in the present study, *S. cerevisiae*, has previously been immobilized in polyacrylamide with minimal decrease in cell viability (Chibata *et al*, 1974; Tosa *et al* 1974); and has also been immobilized for use in metal bioaccumulation processes (Frischmuth *et al*, 1990).

Immobilized biomass can be used to fill a column to produce a fixed-bed reactor. Fixed-bed systems have previously been utilized for wastewater treatment and metal cation accumulation, such as the use of activated carbon to accumulate mercuric ions (McKay and Bino, 1990). Uranium has been recovered from both fresh and sea water by *Streptomyces viridochromogenes* and *Chlorella regularis* trapped in polyacrylamide (Nakajima *et al*, 1982).

IMMOBILIZATION OF YEAST FOR BIOACCUMULATION

Yeast (*S. cerevisiae*) cells in the free state are the most efficient for biosorption owing to their high surface area, however they cannot tolerate repeated biosorption/desorption cycles. Immobilized yeast cells have a better mechanical strength than free cells and are therefore better suited for use in columns for industrial use (Larsson *et al*, 1991). The cells must be immobilized in such a way that they have the greatest possible contact with the wastewater stream. Immobilized columns of *S. cerevisiae* in mercury contaminated wastewater streams were seen not only to accumulate the metal but also, in the long term, to serve as a substrate for mercury resistant bacteria which in themselves accumulated mercury during population growth (Brunke *et al*, 1991).

4.2 MATERIALS AND METHODS

4.2.1 Immobilization:

The method of immobilization used was that of Chibata *et al* (1974, 1986) as follows:

Cell suspension: 10 g wet mass of *S. cerevisiae* was suspended in 20 cm^3 physiological saline (0.15 $mol.dm^{-3}$ NaCl) at 8°C.

Monomer: Acrylamide monomer (7.5 g) plus *N,N'*-methylene-bisacrylamide (0.4 g) was dissolved in 24 cm^3 deionized water and cooled to 8°C.

Immobilization: The above monomer solution and cell suspension were thoroughly mixed together. Added to this mixture was 1 cm^3 of 2.5% TEMED (*N,N,N',N'*-tetramethylethylenediamine), a catalyst for free radical propagation during the polymerization process, and 5 cm^3 of 1% ammonium persulphate which initiates the process. The temperature of the solution/suspension was maintained below 50°C during the

exothermic polymerization process so as to not damage the biomass.

4.2.2 Column Preparation:

The immobilized biomass was passed through a 30 mesh (500 μm) sieve as described by Nakajima *et al* (1982). This yielded thin threads of yeast cell containing polyacrylamide gel, 5 g wet mass of which was subsequently placed in deionized water and then poured as a slurry into a chromatography column.

4.2.3 Experimental Conditions:

Flow rate: 1 $cm^3.min.^{-1}$. Fraction volume: 10 cm^3. Column height: 10 cm. Column volume: 20 cm^3. Temperature: 20°C ± 2°C.

Metal stock solutions:

(a) 200 μmol.dm^{-3} solution of metal chloride in 5 mmol.dm^{-3} HEPES buffer pH 7.2 (Highveld Biological Ltd).

(b) 200 μmol.dm^{-3} solution of metal chloride salt (unbuffered).

Metals: $CuCl_2$ (Merck), $CdCl_2.H_2O$ (Merck), $CaCl_2$ (PAL), $CoCl_2.6H_2O$ (Merck). EDTA solution for cation elution after bioaccumulation: 30 cm^3 of 1.0 mmol.dm^{-3} EDTA in 5 mmol.dm^{-3} HEPES buffer pH 7.2. Buffer (30 cm^3) was used to wash the column before and after the EDTA elution procedure. HEPES and PIPES buffers have overlapping buffering ranges and neither bind heavy metals (Good *et al*, 1966), thus either may be used in metal binding studies at neutral and near neutral pHs.

4.3 RESULTS

The bioaccumulation preference sequence was Cu > Co > Cd. The level of metal accumulation for each metal at neutral and slightly acidic pHs is presented in figures 4.1 to 4.3 and summarised in table 4.1.

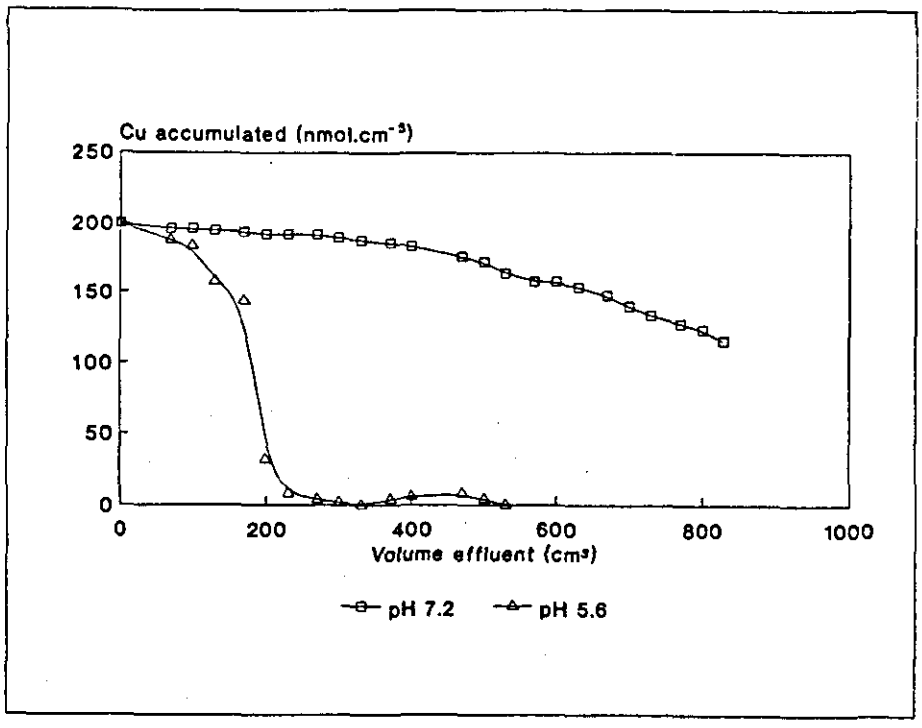


Figure 4.1: Copper accumulation by a column packed with polyacrylamide immobilized yeast cells. Influent pHs were 7.2 and 5.6

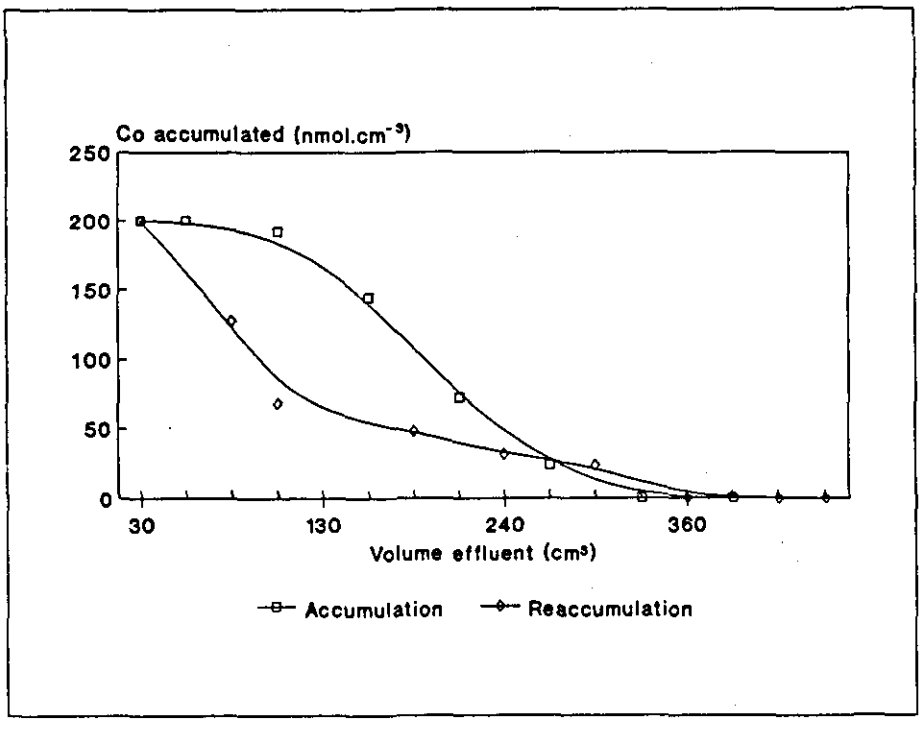


Figure 4.2: Cobalt accumulation by a column packed with polyacrylamide immobilized yeast cells. Influent pHs were 7.2 and 6.0.

Each metal was accumulated to a lesser degree at the lower pHs of the unbuffered metal salt solutions. This possibly reflects hydrogen ion competition or reduced hydrogen flux across the membrane. Copper accumulation was most severely affected by low pH.

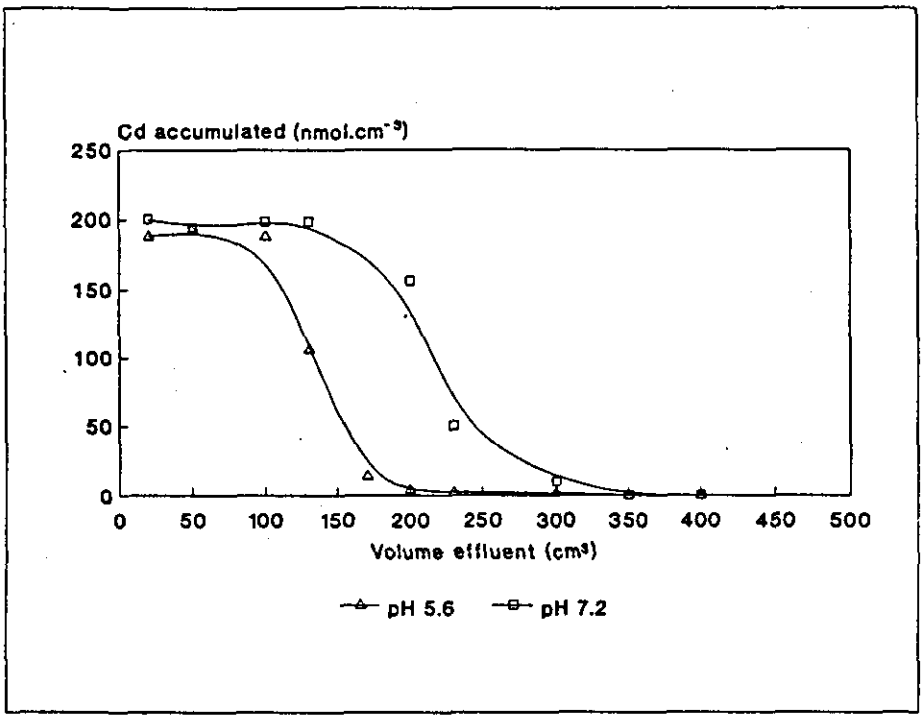


Figure 4.3: Cadmium accumulation by a column packed with polyacrylamide immobilized yeast cells. Influent pHs were 7.2 and 5.6.

TABLE 4.1: Total accumulation of selected heavy metals by polyacrylamide immobilized yeast.

Influent pH during accumulation	Metal bioaccumulation (nmol metal per mg dry mass yeast)		
	Cu	Co	Cd
7.2	480	100	133
5.6 - 6.1	110	87	87

Re-uptake was possible with all three metals investigated after washing with the chelator EDTA. Re-uptake levels were similar for Cu, but Cd and Co re-uptake were reduced by approximately one third (figures 4.4 to 4.6). There appeared to be negligible biomass loss from the system into the effluent as measured by absorption at 540 nm (data not shown).

Addition of Ca^{2+} to the medium failed to modify copper uptake (figure 4.7). During copper accumulation in the presence of calcium the effluent calcium concentration exceeded the influent calcium, suggesting calcium loss may be related to the accumulation process in some way. Later experiments showed that calcium release during

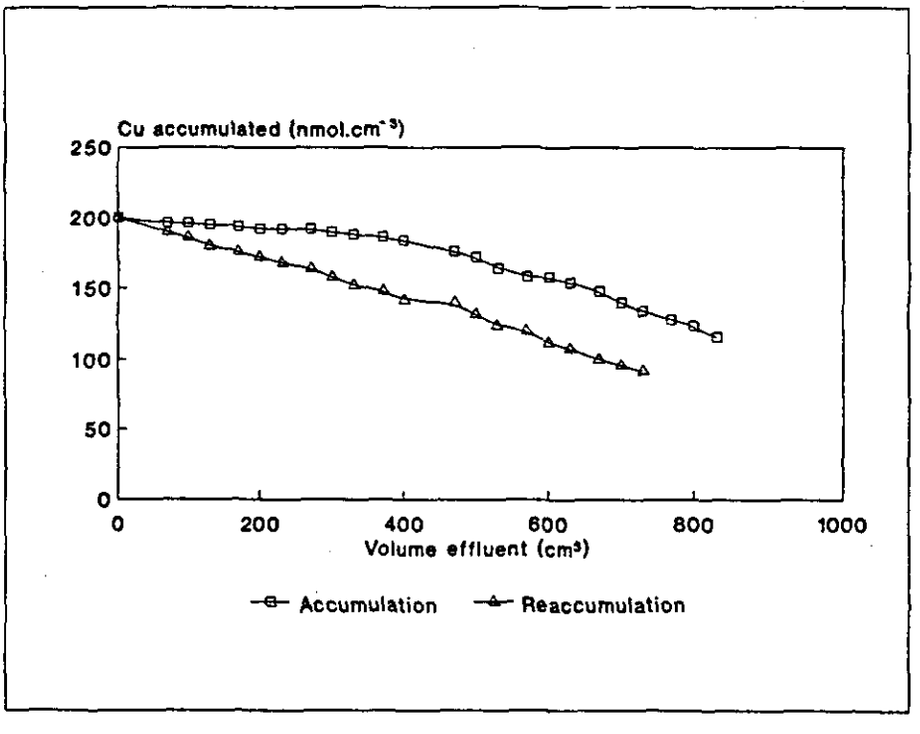


Figure 4.4: Copper accumulation and re-accumulation by a column of immobilized yeast cells. After accumulation the biomass bound metal was eluted with an EDTA solution and washed with ultra-pure water. This was followed by a second accumulation cycle.

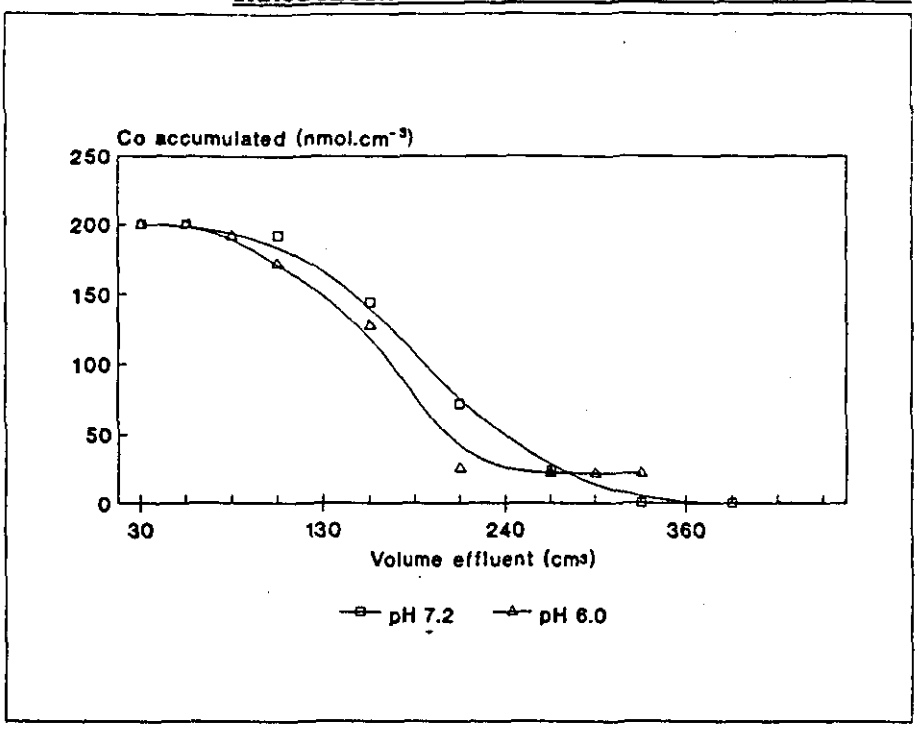


Figure 4.5: Cobalt accumulation and re-accumulation by a column of immobilized yeast cells. After accumulation the biomass-bound metal was eluted with an EDTA solution and washed with ultra-pure water. This was followed by a second accumulation cycle.

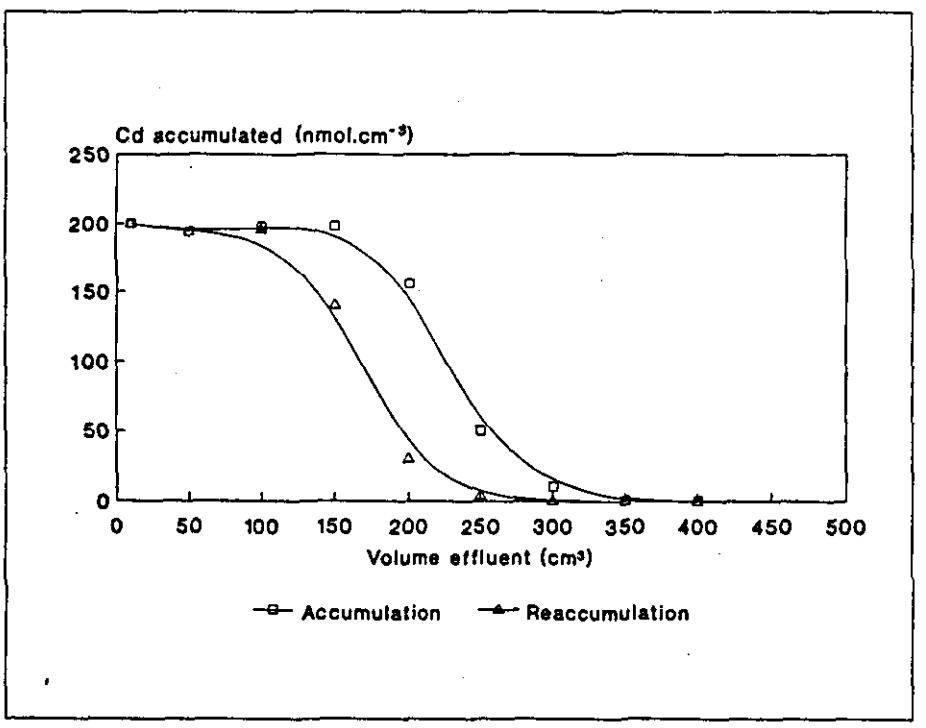


Figure 4.6: Cadmium accumulation and re-accumulation by a column of immobilized yeast cells. After accumulation the biomass-bound metal was eluted with an EDTA solution and washed with ultra-pure water. This was followed by a second accumulation cycle.

cadmium accumulation appears to be very closely related to the ability of the biomass to accumulate cadmium at pH 7.2 (figure 4.8) and 5.6 (data not shown). The calcium may be released from either the cell surface or the interior as these experiments were not designed to differentiate between the two.

Copper bioaccumulation efficency is dependent on the ambient copper concentration. As can be seen in figure 4.9 a 1 mmol.dm^{-3} Cu solution exceeds the ability of the immobilized biomass to completely remove copper from solution, even though the total capacity of the biomass has not been exceeded. This may signify that some of the bioaccumulation mechanisms are not sufficiently rapid to compensate for the higher metal concentration. Alternatively,limits to the rate of diffusion of Cu^{2+} into the polyacryamide gel may be responsible for this effect.

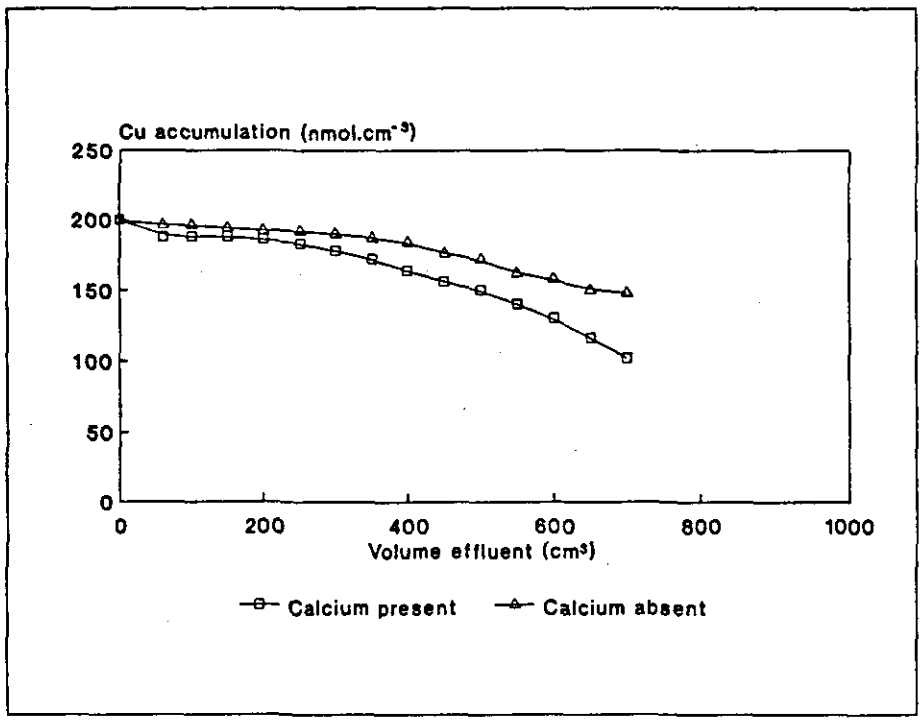


Figure 4.7: Copper accumulation during calcium competition by a column packed with polyacrylamide immobilized yeast. Influent pH was 7.5.

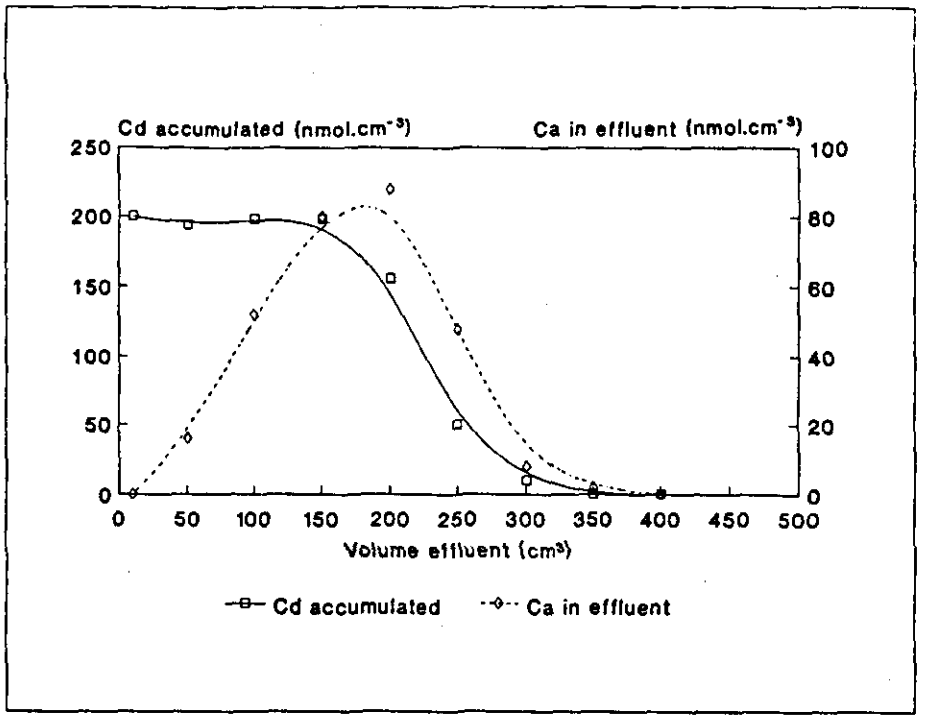


Figure 4.8: Calcium release from a column packed with polyacrylamide immobilized yeast cells during accumulation of cadmium. Influent pH was 7.2.

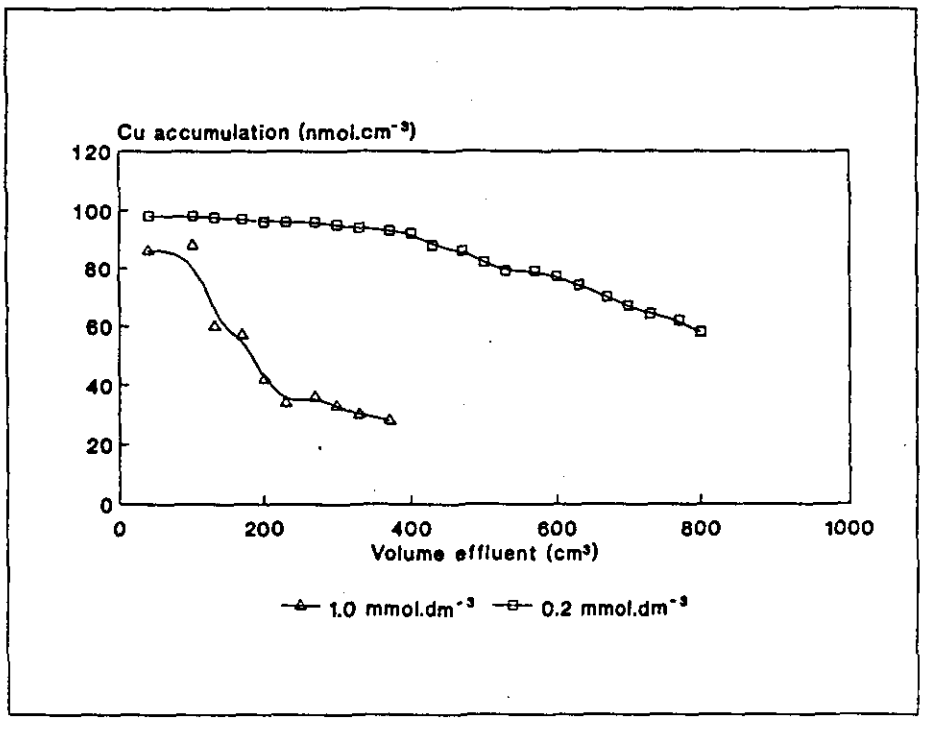


Figure 4.9: Copper bioaccumulation at two influent copper concentrations (1.0 mmol.dm^{-3} and 0.2 mmol.dm^{-3}) by a column of polyacrylamide immobilized yeast cells.

In these experiments it was determined that none of the metal cations studied were accumulated by the polyacrylamide gel alone. This is in agreement with the results of Darnall *et al* (1986) who found that polyacrylamide alone did not retain Cu^{2+}, Au^{3+} or Zn^{2+}, but Hg^{2+} was to a degree retained.

4.4 DISCUSSION

The advantages of cell immobilization include easier separation of the cells from the reaction mixture, improved levels of biomass reuse, high biomass concentrations, and achievement of high flow rates. Unlike certain membrane systems, there may be only very limited clogging in continuous flow systems involving immobilized biomass (Gadd, 1990a), although limits to rates of diffusion may present a problem.

The calcium loss observed in the present study during bioaccumulation may have a practical advantage as calcium loss rather than uptake of individual metals could be monitored as an indication of the metal accumulation by the system. Calcium in the effluent minus that in the influent could possibly be integrated to give an approximate indication of column saturation. This would require a single monitoring sytem for calcium

Immobilized *Rhizopus arrhizus* biomass accumulated Cd^{2+}, Cu^{2+}, Fe^{3}, Mn^{2+}, Pb^{2+}, and Zn^{2+}. The quantity of cadmium accumulation was determined by a number of external factors. Low pH, low temperature, and the presence of competing cations and complexing agents diminished the

level of accumulation but low levels of organic compounds and alkali metals did not affect the system. The biomass could also be reused after regeneration with 0.1 N HCl as an eluting agent (Lewis and Kiff, 1988).

It appears from the present results that metal uptake is dependent on metal concentration (see chapter 2). However, if we look at the column experiments we obtain > 99% removal of metal from solution. The following model of bioaccumulation in column systems may serve as an explanation for this. Column systems behave according to theoretical equilibrium plate theory and could therefore be capable of removing metal cations more completely from solution than would a free cell suspension which would permit only a single equilibration. Consider a fixed bed column filled with a bioaccumulator. As the influent metal-contaminated wastewater enters the column it equilibrates with the biological packing, this requires a certain depth of material called a theoretical plate, and here perhaps 55% of the available metal is accumulated by the packing (a figure extracted from actual equilibrium results, table 2.1). As the liquid leaves the first theoretical plate it encounters a second plate, and perhaps 55% of the remaining metal is retained. The results a number of repetitions of this process can be seen in table 4.2.

The limitation of the efficiency of bioaccumulation is therefore only determined by the physical size of the column. The theoretical plate lengths depend on such factors as the biomass type, the diffusion coefficients in the immobilized biomass, the metal ion species and concentration, and the flow rate.

TABLE 4.2: Metal cation accumulation by a theoretical bioaccumulation column.

Theoretical plate level	metal accumulation %	Metal concentration in effluent ($\mu mol.dm^{-3}$)
1	55	200
2	55	90
3	55	40.5
4	55	18.2
5	55	8.2
6	55	3.7
7	55	1.7
8	55	0.8
9	55	0.3
10	55	0.15

Influent metal concentration: 200 $\mu mol.dm^{-3}$.
Total uptake 99.85%
Effluent metal concentration 0.15 $\mu mol.dm^{-3}$.

A possible method of immobilization which has not been used in these experiments, but has potential advantages, is binding of cells to inorganic or organic supports utilizing metal-cross-link processes. This technique has been successfully utilized by Cabral *et al* (1986) and Vijayalakshmi *et al* (1979) to immobilize *S. cerevisiae* cells. The simultaneous binding of the cells to the support with onset of bioaccumulation would be advantageous, and a metal recovery process could be envisaged in which elution of the metal from the cells would release the cells, and only those capable of re-binding metal would be re-attached to the support for further accumulation cycles, while old and degraded cells would be selectively removed. This process has not been attempted to date but, should it succeed, it would represent a major advance in the field.

4.5 CONCLUSIONS

In conclusion, immobilization of biomass to be used in heavy metal bioaccumulation would have many advantages as it allows for complete removal of heavy metals from solution and recycling of the biomass. The present challenge is to develop a method of immobilization that is both inexpensive and does not cause a decrease in the bioaccumulation capacity of the biomass, while allowing for effective recovery of the metal and reuse of the biomass. Finally the system may allow for selective removal of damaged biomass as proposed above.

The development of a system with numerous sequential equilibria, such as a column system, allows for practically complete removal of metals. The conformation of the bioaccumulation system can therefore modify the level of bioaccumulation to an enormous degree.

The use of immobilized biomass in conjunction with other processes, such as microfiltration systems, may allow for extremely effective bioaccumulation processes.

In chapter 2 the effects of differences in the extracellular solution on bioaccumulation were investigated, while in chapters 3 and 4 the differences in the type of biomass retention system were investigated. The third variable in the bioaccumulation process is the biomass itself. Does the cell's total capacity for heavy metal bioaccumulation change after exposure to metals? This question will be discussed in the next chapter.

5. METAL TOLERANCE IN YEAST

5.1 INTRODUCTION

Microorganisms are known to have an ability to acquire tolerance to toxic levels of chemicals such as antibiotics and heavy metals (Mergeay, 1991). It is conceivable that the acquisition of tolerance to toxic metals by yeasts would be of use in metal bioaccumulation processes. Such tolerance could be through modification of the cell wall components to accumulate more metal ions and thereby protect the cytoplasm and plasmalemma; alternatively,the microorganism could produce large quantities of metal-sequestering proteins such as metallothionein for much the same reasons.

Heavy metals can be as toxic to yeasts as they are to other life forms. Copper can for instance affect yeast growth (Imahara *et al*, 1978). Yeasts have previously been shown to acquire resistance to toxic metals such as copper (Ross and Walsh, 1981), cadmium (Macara, 1978) and cobalt (Perlman and O'Brien, 1954). In the laboratory this requires either serial culturing of the yeast in liquid cultures or on solid media containing sequentially higher levels of the metal in question, or using a continual culture in which a slow increase of metal concentration is added to the growth medium.

Research Aims:

The aim of the present study was to develop tolerance in *Saccharomyces cerevisiae* and to determine whether the acquisition of tolerance by *S. cerevisiae* can be related to improved bioaccumulation by the yeast.

5.2 MATERIALS AND METHODS

5.2.1 Culture Conditions: Yeast cells, which had been stored frozen in liquid nitrogen, were plated out and subsequently serially cultured on 6% (w/v) Sabouraud dextrose agar (containing 4% dextrose) in petri dishes and incubated at 30°C. Sabouraud dextrose agar is selective for yeast and was therefore used to select for and maintain axenic yeast cultures. Axenic cultures prepared this way were stored at 4°C. Samples of cultures were plated out on 3.1% (w/v) nutrient agar and incubated at 30°C as a monitor for contamination by unwanted microorganisms; none were observed.

During experiments to induce metal tolerance the isolated yeast was incubated at 30°C in medium consisting of (in $g.dm^{-3}$): KH_2PO_4, 2.72; K_2HPO_4, 5.22; $(NH_4)_2SO_4$, 1.98; $MgSO_4.7H_2O$, 0.12; $FeSO_4.7H_2O$, 0.0022; D-glucose, 10.0; and yeast extract, 1.0. The D-glucose and the yeast extract were sterilized by filtration through Millipore 0.45 μm filters and added to the growth medium which had been autoclaved separately (121°C, 20 min). Erlenmeyer flasks (250 cm^3) containing 50 cm^3 of growth medium were inoculated with yeast and shaken (100 rpm) for 24 hours. An identical quantity of cells (equivalent to 1.69 mg dry mass.cm^{-3}) was included in each flask, by reference to an extinction-cell dry mass correlation standard curve (Appendix 3). Added to this medium were varying concentrations of $CuCl_2$ solution (0 - 10 mmol.dm^{-3}). The cultures were sequentially transferred from one 24-hour culture to fresh medium which contained either equal of greater concentrations of copper salts. Growth was determined spectrophotometrically at 540 nm using a Bausch and Lomb Spectronic 1001 spectrophotometer.

5.2.2 Critical Point Drying Technique for Cell Preparation for Scanning Electron Microscopy:

A yeast cell suspension was filtered through a Millipore Unipore polycarbonate membrane filter (0.2 μm pore size) and the filter disc with the retentate cells was then folded and fastened shut with a pin to retain the cells through following washes. The filter disc was submerged in cold buffered fixative (2.5% gluteraldehyde in 0.1 $mol.dm^{-3}$ phosphate buffer) for 12 hours. This was replaced with cold phosphate buffer and washed twice with the same buffer (15 min). The filter disc was then dehydrated for 10 min in a sequence of 30%, 50%, 80%, 90%, and 100% (v/v) ethanol solutions (the last of which was repeated). This was replaced by a 75 : 25 ethanol : amyl acetate mixture for 15 - 20 min. This step was repeated with 50 : 50 and 25 : 75 mixtures of the same solvents, finally being replaced by a pure amyl acetate wash which was allowed to permeate the sample for 20 min. This solution was replaced with fresh amyl acetate after transfer to a critical point drying basket and basket holder, which in turn was placed in a critical point drying chamber and dried. The specimen was stored under dessicating conditions until sections of the filter disc and adherent cells were mounted on metal stubs (thick disks of metal that are used to carry the cells through the subsequent processes). The stubs were gold coated in a sputter chamber and subsequently viewed with a JEOL JSM-80 scanning electron microscope.

5.2.3 Copper Bioaccumulation Studies:

Bioaccumulation of Cu^{2+} by the Cu^{2+}-tolerant and parent (native) yeasts was investigated using the method of Norris and Kelly (1977), as used in chapter 2.

5.3 RESULTS

The general trend found was of decreased cell growth with increased copper concentrations in the medium (table 5.1). Repetitious growth in medium containing the same copper concentration resulted in improved growth, arguing for a mechanism of tolerance by adaption.

Scanning electron microscopy of *S. cerevisiae* shows the cells to be an average 5 μm in diameter, being slightly eliptical; the surface is smooth except for bud scars (figure 5.1a). After exposure to copper *ion-containing solutions* (200 μmol.dm^{-3}) the surface of copper-tolerant yeast becomes convoluted, the cell is generally more spherical and somewhat smaller, being 3 μm in diameter (figure 5.1b).

Bioaccumulation of copper by copper-tolerant cells as compared to the parent culture can be seen in figure 5.2. In each case the cell concentration was 0.4 mg yeast dry mass.cm^{-3}. The copper-tolerant cells accumulated less metal than the parent strain. Moreover, it is apparent that although the initial accumulation within the first five minutes is similar in both copper tolerant and parent strains, the tolerant strain, unlike the parent strain, does not accumulate any further metal. This suggests a mechanism preventing metal uptake, which in turn implies that the tolerant cell either limits copper accumulation by modifications to the cell wall, cell membrane, or to the cation ports in the membrane.

Table 5.1: Growth of *S. cerevisiae* after 24 hours of incubation in copper-containing growth medium.

Cu^{2+} conc. $\mu mol.dm^{-3}$	Absorbance 540 nm	
	Sample flask*	Cu^{2+}-free control
0	1.793	-
10	1.447	1.656
20	1.712	1.635
50	1.729	1.854
100	1.780	1.840
200	1.470	1.545
200(R)	1.880	1.758
300	1.808	1.795
500	1.593	1.820
500(R)	1.798	2.103
600	1.449	1.508
700	1.754	1.932
700(R)	2.184	2.559
800	2.062	2.427
900	1.414	1.620
900(R)	1.957	2.055
950	1.764	1.919
1 000	1.591	1.765
1 000(R)	1.850	2.070
1 500	1.406	1.895
2 000	1.337	1.977
2 000(R)	1.183	1.273
3 000	1.000	1.311
5 000	1.135	1.394
5 000(R)	1.668	1.821
7 000	1.433	1.929
10 000	1.585	2.013

(R): Repetition of concentration in sequential culturing.
* The sample flasks were blanked at 540 nm with cell-free medium including the appropriate copper concentration.

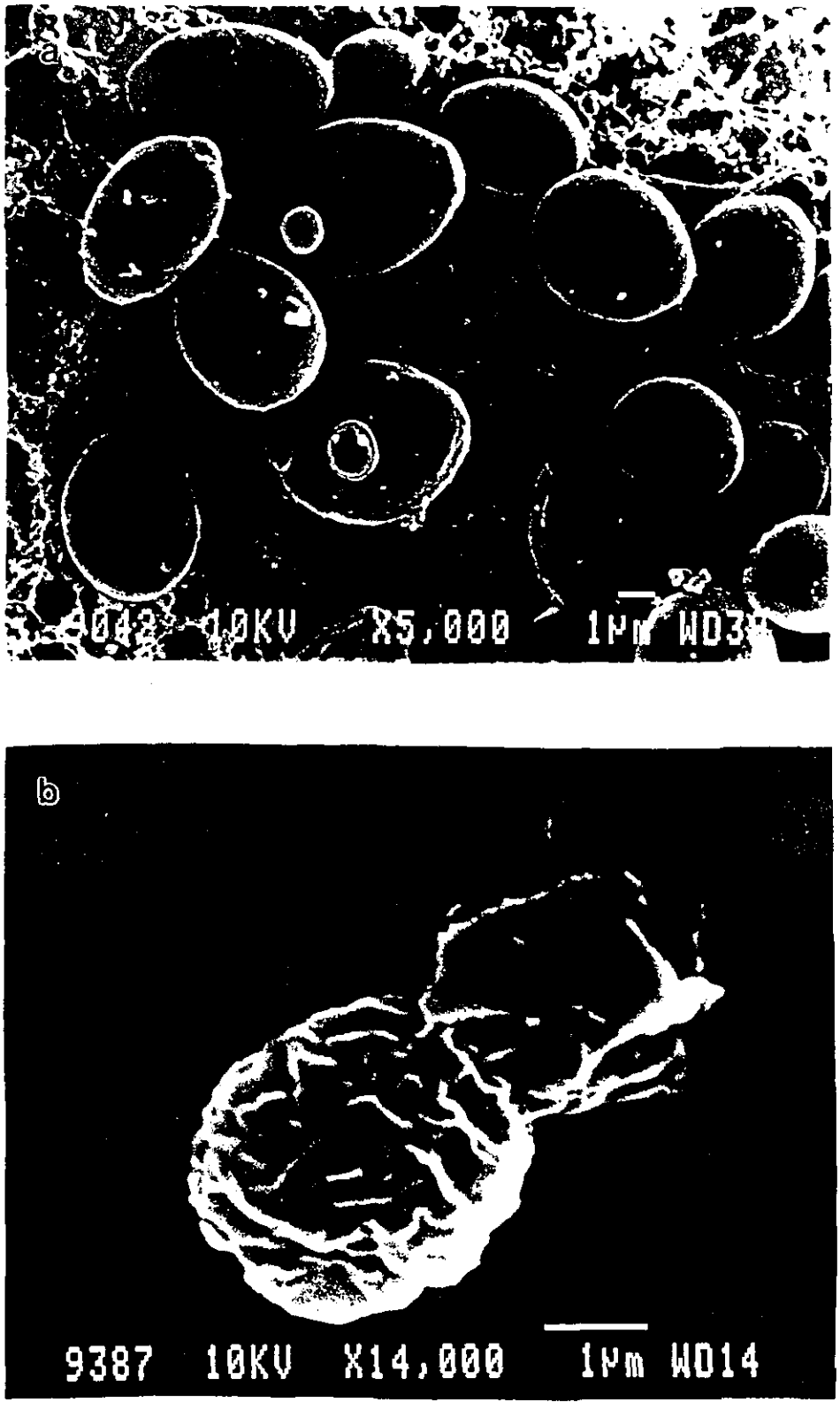


Figure 5.1: Scanning electron micrographs of parental *S. cerevisiae* cells (a) and copper tolerant *S. cerevisiae* cells after incubation with high levels of copper chloride (b).

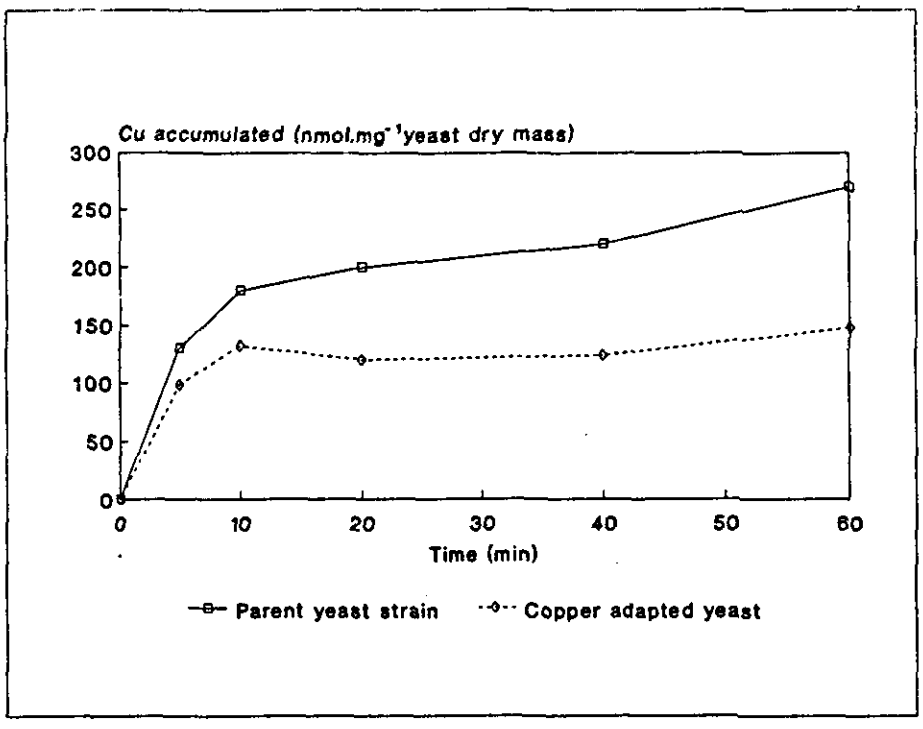


Figure 5.2: Accumulation of copper by native and copper tolerant yeast cells. The value plotted for each sample represents the average of four replicates.

5.4 DISCUSSION

5.4.1 Development of Heavy Metal Tolerance in *S. cerevisiae*:

Certain metals are referred to as toxic and others as nutrients, while some appear to be nutrients when encountered in low concentrations and cause toxic effects at higher concentrations. All metals, however, appear to be capable of antagonism and inhibition of cell metabolism and growth at appropriate concentrations (Jones and Greenfield, 1984) and therefore most of them can realistically be regarded as toxins at appropriate concentrations.

In the present study a strain of *S. cerevisiae* developed tolerance to high ambient concentrations of copper cations which would otherwise be toxic to it, either by environmental selection for mutants or possibly the development of resistance. The terms "tolerance" and "resistance" are often used in the literature without clear distinction. Gadd (1990b) suggests that "resistance" should be used to describe a direct response of the organism subsequent to heavy metal exposure.

(a) Tolerance to copper:

Ross and Walsh (1981) selected a genetically copper tolerant yeast by shock dosage of the growth medium with copper. Tolerant yeast could grow in conditions of 75 - 100 $\mu mol.dm^{-3}$, whereas normal cell growth was reduced significantly at 25 $\mu mol.dm^{-3}$ copper. The resistance of the parent yeast gained by serial adaption to copper was rapidly lost after transfer to copper free medium, while the copper resistance of the genetically resistant yeast was of a stable nature, even after transfers in copper free media. The authors concluded that yeast have an intrinsic ability to adapt to elevated copper concentrations, which is independent of any genetic resistance traits. This in turn suggests that genetically resistant strains may utilize this adaption process once the limits to their resistance mechanism have been exceeded.

The resistance to copper therefore appears to be due to two distinct mechanisms: (1) Genetic alterations of the yeast, possibly modifying the cation ports to reduce copper absorption, and (2) Adaption to metal, which is less stable and may involve inducible metallothionein-like proteins or changes in the cation transport systems. The

difference is that genetic resistance is permanent, while adaption mediated resistance is temporary.

Copper resistance in certain strains of *S. cerevisiae* has been ascribed to the gene amplification of the CUP 1^R locus which codes for the protein metallothionein, a small copper chelating protein which has abundant cysteine residues (Butt and Ecker, 1987). Other mechanisms of resistance are known. In bacteria there is often a link between heavy metal resistance and resistance to antibiotics and other drugs (Nakahara *et al*, 1977). Some of the bacterial mechanisms of resistance are involved in the detoxification of the environment (Williams and Silver, 1984). Bacterial methods of heavy metal resistance include metal reduction to non-ionic species, efflux of toxic ions and decreased uptake and are usually mediated by plasmids (Mergeay, 1991). The efflux is often directly mediated by ATPase pumps (Silver *et al*, 1989).

The toxic effect of copper also depends on its availability to the cells. Addition of cysteine (which can complex with transition metals) to the growth medium was found to significantly decrease the inhibitory effect of copper on cell growth (Imahara *et al*, 1978).

(b) Tolerance to metals other than copper:

Perlman and O'Brien (1954) studied the tolerance of *S. cerevisiae* to cobalt through serial increases of cobalt concentrations in the growth medium. The yeast was eventually capable of growing in 750 ppm (12.7 $mmol.dm^{-3}$) cobalt-doped growth medium during incubation at 25°C. Under these conditions 9.9 percent of the cell content by mass was cobalt, which was noted to be firmly bound to the cell. The

morphology of the cells was not modified by these high cobalt levels.

Nickerson and Van Rij (1949) on the other hand found that cobalt could inhibit cell division in various yeasts without consequent inhibition of cell growth, resulting in elongated mycelial elements. Similar mycelial growth was observed in the present copper tolerance experiments (scanning electron micrograph not shown), while no contamination of the cultures was observed when grown on nutrient agar.

Macara (1978) noted that temporary adaption of *S. cerevisiae* to cadmium cations occurred during sequential culturing in growth media increasingly contaminated by cadmium. A progressive decrease of cadmium tolerance after serial culturing in cadmium free medium led to the conclusion that the tolerance was adaptive and not the result of a genetic mutation. However,chromatography of the soluble fraction of cadmium adapted yeast on Sephadex G-25 did not reveal the presence of any metallothionein-like protein. It is possible that high intracellular levels of cadmium inhibit the divalent cation transport in yeast.

The adaption of yeast to cadmium can be induced, to a limited degree, by pre-growth of the yeast in 50 μmol.dm^{-3} Zn^{2+} without the presence of cadmium (Macara, 1978).

5.4.2 Relationship of Bioaccumulation of Copper to Copper Tolerance:

Similar to the results found in this study, Ross and Walsh (1981) found that a copper-resistant *S. cerevisiae* strain accumulated less copper than the parent strain under the same conditions. As total cellular

copper was measured it was not possible to tell if this was a result of decreased uptake by the yeast.

Copper toxicity in copper sensitive *S.cerevisiae* has been found to be optimized by addition of glucose to the medium (Ross, 1977). Glucose was seen to induce accumulation of copper at low ambient levels, which suggested a mechanism of uptake dependent on metabolic energy. The probable reason for these facts is that there is an initial energy dependent uptake of copper which rapidly results in a toxic concentration of this metal within the cells. It is therefore possible that the majority of the observed intracellular uptake of copper is due to non-viable cells which can no longer control their permeability to cations (Jones and Gadd, 1990; Gadd 1990a).

The resistance of *S. cerevisiae* to metals appears to have either of two effects - either increased or decreased metal cation accumulation, depending on the strain of yeast studied and the species of metal involved. In the present study resistance of *S. cerevisiae* to copper involved reduced uptake of the metal. This type of response has been well documented in the literature. Repeated culture of *S. cerevisiae* in elevated levels of copper, cadmium and cobalt salts resulted in increased resistance, and all these adapted strains showed decreased accumulation of these metals (White and Gadd, 1986). Joho *et al* (1985) also showed that a cadmium-resistant strain of yeast was characterized by the decreased uptake of Cd^{2+}, while in other studies cells of a strain of *S. cerevisiae* which was not cadmium-resistant accumulated up to four times as much cadmium as a resistant strain of the same species (Belde *et al* (1988); Birch and Bachofen, 1990).

However, resistance does not always result in reduced accumulation of metals. Several *S. cerevisiae* mutants which are resistant to Mn^{2+} have been found and exhibit increased uptake of Mn^{2+} (Bianchi *et al*, 1981). Results of studies on cadmium-tolerant cultures of yeast often reveal a greatly increased proportion of Cd^{2+} bound in the cytosol (Joho *et al*, 1985).

Another means of resistance appears to be the ability of yeasts to precipitate metals such as copper as sulphides in and around cell walls while other microbes precipitate metals at the cell surface as oxides or phosphates (Gadd, 1990b). These precipitation reactions are probably largely fortuitous and therefore should not be considered heavy metal resistance mechanisms even though they do limit metal toxicity to the cells. This process, again fortuitously, results in extracellular accumulation of metals.

5.5 CONCLUSIONS

In conclusion, metal-tolerant yeasts often accumulate less than the native yeasts. In the processes proposed in this study waste yeast is to be utilized, and thus strain selection would be almost impossible. Regarding the bioaccumulation processes it would appear that native (not metal-tolerant) yeast cells are of greater potential value due to their larger potential bioaccumulation capacity for metal.

PART 2: THE MECHANISM OF BIOACCUMULATION

6. ACCUMULATION OF METAL IONS BY YEAST CELL WALLS

6.1 GENERAL INTRODUCTION

6.1.1 Metal Binding by Microbial Cell Walls:

Exchange of ions with lower affinity for the binding site occurs at the cell surface, such as nickel replacing magnesium on *Pseudomonas* cell surfaces (Bordons and Jofre, 1987). Binding of metals to microbial cell walls has been observed directly by transmission electron micrographs (Beveridge and Murray, 1976; Beveridge, 1986).

Microbial cell walls tend to have an overall negative charge (Beveridge, 1986; Shaeiwitz *et al*, 1989). The surface charge is caused by dissociation of chemical groups, such as carboxyl groups, on the cell wall. Negatively charged groups found on the polymers that constitute the cell wall allow for cation exchange, while other chemical groups allow coordination of metal cations (Blundell and Jenkins, 1977). The exogenous binding of metal cations to the cell wall is both rapid and reversible (Rothstein and Hayes, 1956).

Isolated cell walls of *S. cerevisiae* were found to be capable of binding calcium cations (Ca^{2+}), the more flocculent strains binding more calcium that the less flocculent strains (Lyons and Hough, 1970). The cell walls of *S. cerevisiae* are also capable of binding a wide range of heavy metal ions (Rothstein and Hayes, 1956).

The cell walls of all organisms investigated are mobile in electric fields due to the overall negative electrical charge on the cell wall at neutral pH (Neihof and Echols, 1973). The mobility of the walls decreases with increasing ionic strength suggesting that ions can neutralise the charge on the cell wall and that charged groups are involved in metal cation binding. The mobility of *S. cerevisiae* cell walls in an electric field remained fairly constant over a pH range of 3 - 11 (Neihof and Echols, 1973), which implies that ion exchange groups are not neutralized over this pH range. At very low pH the charge on the yeast cell wall is maintained by phosphate groups, mostly those present as phospho-diester bonds (Neihof and Echols, 1973).

The mobility of the yeast cell walls in an electric field is lower than that of the cell walls of certain other microorganisms such as *Bacillus subtilis* (Neihof and Echols, 1973), suggesting that they have a lower net negative charge, possibly owing to limited numbers of charged groups. This is suggested by evidence that *S. cerevisiae* cells also accumulate less heavy metals than do *B. subtilis* cells (Nakajima and Sakaguchi, 1986). It has been suggested that only a small percentage of the yeast cellular surface material binds exogenous cations (Rothstein and Hayes, 1956).

6.1.2 Kinetics of Metal Binding to Cell Walls:

The binding of cations to the cell wall is an equilibrium reaction. The equilibrium of association between cations and the anionic ligands on the cell wall can be stated simply as such (Rothstein and Hayes, 1956):

$$[M] + [L] \rightleftharpoons [ML] \qquad \ldots\ldots (1)$$

Where [M] represents the concentration of metal ion in solution, [L] represents the concentration of free ligands and [ML] represents the concentration of cation bound to the cell. The mass-law equation of this reaction is as such:

$$K = \frac{[M].[L]}{[ML]} \times \frac{f_M.f_L}{f_{ML}} \qquad \ldots\ldots (2)$$

Where K is the dissociation constant and f_M, f_L and f_{ML} are the respective activity coefficients. It is assumed that the binding of a metal ion to a ligand is independent of binding at other ligands on the cell wall.

The value of f_M can be derived from the metal ion concentration, but the coefficients of the two solid phases are not so easily obtained. This difficulty can be avoided by assuming that the ratio of f_L/f_{ML} is constant. This ratio may be replaced by a single activity coefficient K_1 to yield a new equation (Rothstein and Hayes, 1956):

$$K_1 = ([M].[L]/[ML]) \times f_M \qquad \ldots\ldots (3)$$

Therefore the mass law can be applied to cation binding to the cell wall even though the activity coefficients of the solid phases are unknown. The value K is a ratio between the forward and reverse reaction rates. It is almost independent of the nature of the ligand, but depends strongly on the particular metal ion involved (Jones and Greenfield, 1984).

The total concentration of binding sites can be determined from a Scatchard plot as the intercept at the X axis, or from equation (4).

If $L_t = [L] + [ML]$ then from equation (3) then:

$$\frac{[ML]}{[M]} = \frac{f_M L_t}{K_1} - \frac{f_M [ML]}{K_1} \qquad \ldots\ldots (4)$$

where L_t is the total concentration of binding sites (in $mol.dm^{-3}$) which can be deduced by extrapolation. This equation resembles that originally used by Scatchard to characterize the binding of ions to proteins (Scatchard, 1949; Rothstein and Hayes, 1956).

If the mass per volume of cell wall in suspension is known, then this equation can be modified by converting the concentrations to the specific molar quantity of metal bound per mass of cell wall. The equation is then plotted so that the amount of bound metal per mass of cell wall divided by the concentration of free metal is related to the amount of bound metal divided by the mass of the cell wall (μmol metal per mg cell wall) can be plotted. The intercept of this Scatchard plot with the abscissa now represents the number of binding sites, while the slope of the graph reflects the association constant.

The equilibrium constant for the formation of a divalent metal complex with a ligand can be represented as (Jones and Greenfield, 1984):

$$K_a = \frac{[ML^{2-n}]}{[M^{2+}]\ [L^{n-}]} \qquad \ldots\ldots (5)$$

K_a is the association constant (the inverse of the dissociation constant above, equation (2)), and n is the charge on the ligand. Dissociation of the heavy metal salt is assumed to be complete, and f_M is therefore unity.

For example, the chelating of a metal by the chelating agent EDTA:

$$[M^{2+}] + [L^{4-}] \rightleftharpoons [ML^{2-}] \qquad \ldots\ldots (6)$$

the association constant of which would be:

$$K_a = \frac{[ML^{2-}]}{[M^{2+}]\ [L^{4-}]} \qquad \ldots\ldots (7)$$

The equation presented here is somewhat of a simplification, however. The binding process may consist of a number of steps similar to the one above as it is possible for a multivalent metal to bond with more than one ligand (i.e. to be chelated). The general reaction can therefore be stated as:

$$[ML_{n-1}] + [L] \rightleftharpoons [ML_n] \qquad \ldots\ldots (8)$$

and the association constant for this step is:

$$K_n = \frac{[ML_n]}{[ML_{n-1}]\ [L]} \qquad \ldots\ldots (9)$$

where (n) is the number of ligands. The different steps in the production of ML_n are not easily distinguished experimentally. A cumulative equation of these steps to describe the complete reaction

is therefore more practical, *viz*:

$$[M] + [nL] \rightleftharpoons [ML_n] \qquad \ldots\ldots (10)$$

Binding may be modified by inhibition (e.g. by cation competition for binding sites) and the constant determined by experiments in the presence of co-ions would therefore be described as an apparent binding constant (Jacobson *et al*, 1977).

6.1.3 Metal Cation Binding Ligands:

Group I and II elements (alkali and alkaline-earth metals) form primarily ionic bonds with ligands. The metals in these groups, especially group I, are very electropositive with low ionization potentials for the valency electrons, and there is a complete transfer of electrons from the ligand to the metal (Blundell and Jenkins, 1977). Ion-exchange resins bind metals to "hard" ligands, such as sulphonate or carboxylate groups. This binding is the result of electrostatic attraction of the ionic ligands and the free ions. e.g. Ca^{2+}, Mg^{2+}, and to a lesser extent, Fe^{2+} bind to ligands in the order:

$RCOO^- > RHN^- > RS^- > H_2O$ (Blundell and Jenkins, 1977).

Heavy metal ions are, however, relatively unattracted to these hard ligands compared to the attraction experienced by the alkali and alkaline earth metals, and do not form ionic bonds readily. Binding tends to be covalent where the metal and the counter ion each contribute an electron to the bond. The heavy metals prefer to bind to the "softer" ligands, such as sulphur and nitrogen. e.g. Cu^{2+}, Ni^{2+}, Co^{2+} bind to ligands in the order $RS^- > RHN^- > OH^- >> H_2O$.
The order of softness of heavy metals is:

$Cu^{2+} > Ni^{2+} > Co^{3+} > Zn^{2+} > Co^{2+}$ (Blundell and Jenkins, 1977).

There is variation of binding affinity within the heavy metal group. For example Cu^{2+} prefers the nitrogens of histidine while Zn^{2+} prefers the sulphydryl groups of cysteine (Jones and Greenfield, 1984).

Biomass contains many of these softer binding sites, and therefore has greater binding affinity for heavy metals than for alkali and alkaline-earth metals. This in turn means that biomass can selectively accumulate heavy metal ions, and with less "leakage" or loss of these ions than is the case with the traditional ion-exchangers. Calcium and magnesium, for instance, compete effectively with heavy metals for electrostatic binding sites on ion-exchangers and, as they are usually found in excess in wastewaters, effectively exclude heavy metals by occupation of most of the sites (Walker, 1991; Blundell and Jenkins, 1977).

The covalent binding of heavy metals to soft ligands may be coordinate, a specific type of covalent bond where the ligand donates both electrons. Chelation occurs where a metal ion is coordinated to two or more distinct ligands on the same molecule (a polydentate ligand), so that there is a ring of atoms including the metal. Chelation of a metal is much stronger than metal complex formation with a simple monodentate ligand (Sulkowski, 1985). Sequestration is the process of forming coordination complexes of an ion in solution, and an ion forming a coordinate bond or a chelate may therefore be said to be sequestered.

6.1.4 Composition of the Yeast Cell Wall:

The previous sub-section described the affinity of metals for various ligands. Of importance to this study is the question of which of these ligands are found in the cell wall and in what concentrations. A detailed knowledge of the composition of the yeast cell wall is required to answer this question.

S. cerevisiae has one of the most well-studied of all the fungal cell walls owing to the abundance of this organism (Phaff, 1963), but models of its structure are incomplete, owing to its complex composition and macromolecular form. The wall is approximately 70 ±10 nm thick and is thought to comprise 25% of the cell dry mass (Bacon *et al*, 1969; Catley, 1988). The cell wall of *S. cerevisiae* consists of a number of polymers (see table 6.1), mostly carbohydrate polymers. A small percentage of the cell wall mass may be comprised of inorganic ions such as Ca^{2+} and Mg^{2+} (Bartnicki-Garcia and Nickerson, 1962).

Table 6.1: Molecular composition of the cell wall of *S. cerevisiae*.

Component	% total dry mass
Glucan	28.8
Mannan	31
Protein	13
Chitin	1
Chitosan	1
Lipid	8.5
Ash	3
Unknown	13.7
Total	100
Nitrogen	2.1
Phosphate	0.3

From Northcote and Horne, 1952; Korn and Northcote, 1960; Volesky, 1987.

The actual metal binding sites on the yeast cell wall have not been characterized beyond debate, however the most likely contenders are protein, chitosan (Muzzarelli *et al*, 1980a, 1980b; Muzzarelli 1977) and the phosphomannans (Lyons and Hough, 1970). Research to date on yeast cell wall components and their role in metal binding will now be reviewed.

(a) Glucan:

Glucan, a polymer of ß(1-3) linked glucose with ß(1-6) branches (Bacon *et al*, 1969; Phaff, 1971, see figure 6.1), is found primarily on the cell membrane side of the cell wall where its main function is maintaining cell rigidity (Bacon *et al*, 1969; Phaff, 1963). Some of the glucan is in a alkali-soluble form (Bacon *et al*, 1969; Phaff, 1963).

During cell wall regeneration, nets are observed to form on the cell membrane; these nets are believed to be composed of primarily glucan with some chitin (Peberdy, 1979), showing the two to be associated in some way.

Glucan has not been considered previously as a primary candidate for heavy metal binding. However, owing to glucan's high percentage in the composition of the yeast cell wall, it may have some role in heavy metal cation binding, even if that role is indirect.

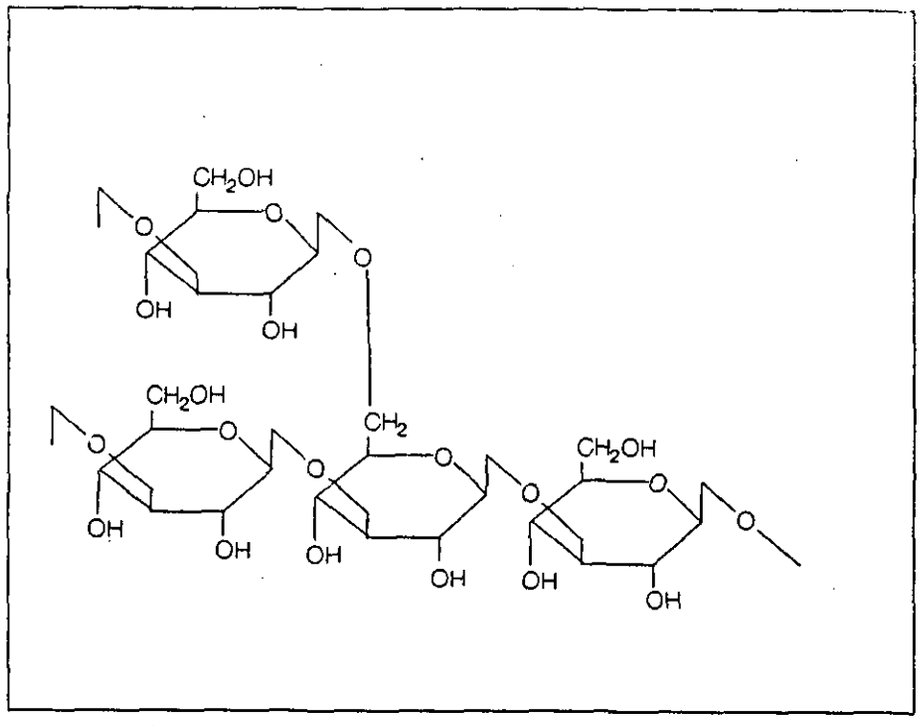


Figure 6.1: The structure of yeast cell wall glucan.

(b) Mannan:

Mannan is a polymer of mannose monomers forming a main chain linked by α(1-6) bonds and side chains with α(1-2) and α(1-3) bonded mannose residues which branch from the main chain via α(1-2) links (Peat *et al*, 1961; Phaff, 1971; see figure 6.2). In yeast the mannan is found as a covalently linked protein-polysaccharide complex of 25 - 500 kDaltons, of which the protein usually contributes 5 - 10%. A number of polymannose chains are joined to the protein backbone *via* short *N*-acetyl glucosamine (NAG) linking chains, which are linked to serine or asparagine side chains of the protein (Phaff, 1971; Ballou, 1974).

$$(\text{mannose})_n\text{-}(\text{NAG}_c)_x\text{-Ser-Protein}$$

$$(\text{mannose})_n\text{-}(\text{NAG}_c)_x\text{-Asn-Protein}$$

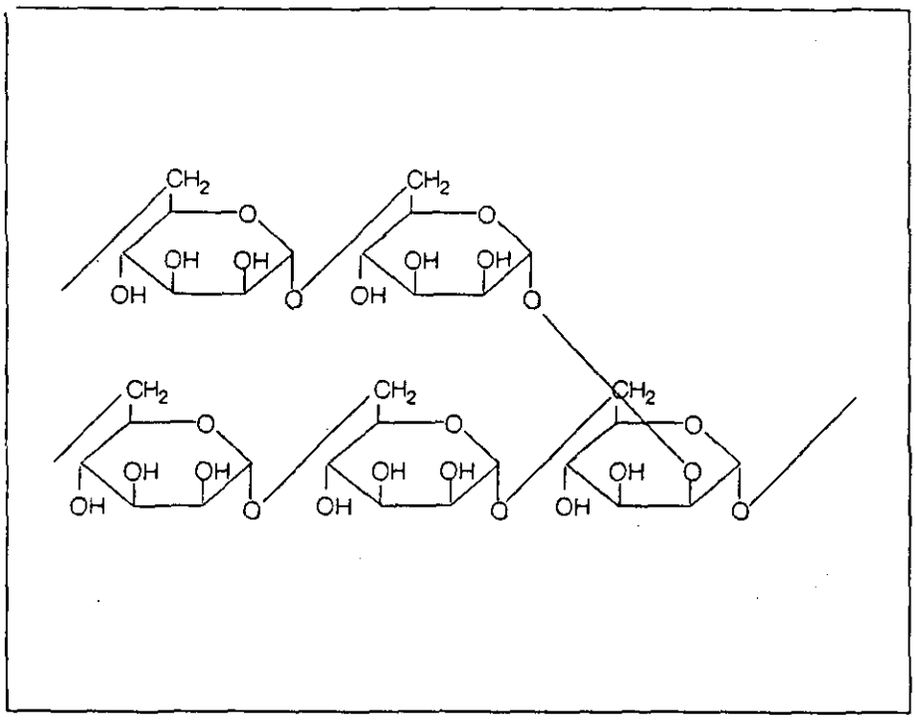
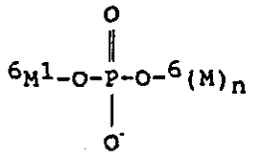


Figure 6.2: The structure of yeast cell wall mannan.

Polymannose branches are cross-linked via phosphate moieties on the mannose residues. The mannan contains approximately 1% phosphate which appears to be linked to the 6-position of the terminal (non-reducing) mannose residue of the main chain, or to the terminal 6-position of the (1-2) or (1-3) linked residues of the polymannose side chains. In the cell wall this phosphate is diesterified, being involved in a hemi-acetal bond to C-1 of another mannose residue (Phaff, 1971), thereby cross-linking the mannan polymers as such:



The main antigenic determinants of *S. cerevisiae* are the $\alpha(1\text{-}2)$ and $\alpha(1\text{-}3)$ mannan side chains which, indicates that mannans are found in the outer section of the cell wall.

Mannans are readily soluble in alkali and may be isolated from the cell by hot alkali treatment. Depending upon the molarity and temperature, the alkali reaction can break the phosphodiester and glycosyl-serine linkages present in the mannan-protein complex (Ballou, 1974). Alkaline degradation can be avoided by cell disruption followed by autoclaving to isolate the cell manno-proteins (Peat *et al*, 1961). Although alkaline extraction hydrolyses the bonds between wall mannan and protein, the isolated manno-protein fraction has a low isoelectric point (Lyons and Hough, 1970), indicating that some protein is still associated with the mannan (see the comments on proteins in the cell wall below).

By complexing with copper cations the mannans can be selectively precipitated out of the alkali solution (Peat *et al*, 1961). This in itself is a good indication that the mannans are the primary copper chelating polymers of the cell wall.

Mannans have also been shown to bind cadmium. *S. cerevisiae* bound up to 0.046 μmol.mg^{-1} cadmium (Oda *et al*, 1988). Most of this accumulation was,however,thought to be through an association with protein and not the carbohydrate moieties because Pronase digestion greatly decreased cadmium accumulation by the mannan (Oda *et al*, 1988). Uranium binding has been related to the phosphate content of the phosphomannan (Strandberg *et al*, 1981).

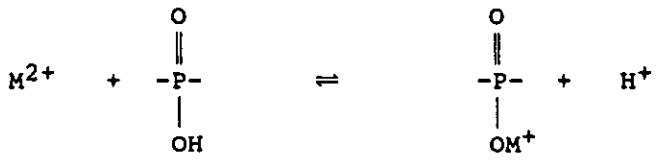
(c) Phosphate:

Phosphomannans are implicated extensively in cation binding in yeast cell walls, and limitation of phosphorus in the yeast growth medium reduces the cation binding potential in the yeast (Lyons and Hough, 1970). The mechanism of biosorption of metals to the cell surface is generally believed to be an ion-exchange process. Cell wall phosphomannan are usually be found complexed to Ca^{2+}, although other cations, if present, can replace calcium (Jones and Greenfield, 1984).

Flocculation of yeast by calcium ions is believed to be due to cross-linking of the anionic cell wall groups of adjoining cells by the divalent ions. There was found to be a greater percentage of phosphate in flocculating yeasts in comparison with non-flocculating strains (Lyons and Hough, 1970; Phaff, 1971) indicating that there was a high percentage of mannan, as the two are intimately associated. Paradoxically, another strain of flocculent yeast had a lower content of mannan than non-flocculent yeast (Phaff, 1963). Flocculent yeasts bind an average of 0.034 $\mu mol.mg^{-1}$ Ca^{2+} per cell wall dry mass, while non-flocculent yeasts binds 0.016 $\mu mol.mg^{-1}$ per cell wall dry mass (Lyons and Hough, 1970). Culturing cells in magnesium-starvation conditions results in a higher phosphorus content in the cells and a greater capacity for cations, while culturing in phosphate-deficient media reduced the wall's Ca^{2+} binding capacity (Lyons and Hough, 1970).

In comparison to yeast, uranium accumulation by the filamentous bacterium *Streptomyces longwoodensis* after freeze-drying was found to be in a stoichiometric ratio with the phosphorus content of the cell, giving a simple 1:1 ratio, while during binding hydrogen ions were released in the same ratio (Friis and Myers-Keith, 1986). The

following model of metal binding was presented by the authors:



Phosphate and carboxylate groups were found to be important moieties in metal binding by *Rhizopus arrhizus*, while the contribution of amine groups was deemed to be low because at the pH of the experiment (pH 4) they would be in a protonated state (Tobin *et al*, 1990).

(d) Protein:

Protein is found throughout the cell wall of *S. cerevisiae* but is more prominent in the outer mannan layer (Korn and Northcote, 1960). In the fungus *Agaricus bisporus*, cystine-containing proteins were also found to occur throughout the cell wall (Muzzarelli, 1977).

The amino-acid composition of one protein fraction of the *S. cerevisiae* cell wall is revealing. The two acidic amino acids glutamic acid (17.8%) and aspartic acid (13.1%) comprise 30.9% of the total amino acids of one of the two major protein fractions of the cell wall. The other protein fraction is composed of even greater quantities of acidic amino acids, i.e. 40.3% (aspartic acid, 31.1% and glutamic acid, 9.2%), (Phaff, 1971). The remainder of the residues are mainly small neutral amino acids. These proteins can therefore be regarded as poly-anionic, and therefore eminently capable of binding cations. In *S. cerevisiae* the protein was also found to have a high sulphur to protein ratio (Phaff, 1963), probably in the form of cystine residues. This high sulphur content appears to be the reason why thiols assist

cell wall degradation (Bartnicki-Garcia and McMurrough, 1971). These cystine residues or reduced cysteine residues could be involved in heavy metal coordination.

Proteins possess a wide range of possible complexing groups with lone pairs of electrons such as those associated with amino groups, carbonyl groups, the diesterified oxygen of peptide bonds, and the nitrogens of histidine residues (Crist *et al*, 1981). As a result bovine serum albumin, for example, may accumulate 0.054 μmol of Cd^{2+} per mg (Oda *et al*, 1988). It has also been found that there is linear relationship between the amount of certain heavy metals accumulated within a cell and the cell protein content (Dedyuhina and Eroshin, 1991).

(e) Chitin and Chitosan:

Chitin:

Chitin is a polymer of N-acetylglucosamine residues linked by ß(1-4) glycosidic links. Chitin is associated with protein in the cell walls (Austin, 1988) to which it is linked via non-aromatic amino acid residues (Muzzarelli, 1977). Cross-linking of chitin increases during the growth cycle (McGahren *et al*, 1984). Chitin may form crystals in the cell walls, especially while binding transition metal ions (Muzzarelli, 1977). Chitin is found as microfibrils in the inner-layer of the cell wall in the glucan matrix (Muzzarelli, 1977).

The dissociation of the amine (see figure 6.3) in solution provides heavy metal coordination sites *via* the lone pair of electrons (Tsezos, 1983):

$$R\text{-}N^{+}\text{:}H + H_2O \rightleftharpoons R\text{-}N\text{:} + H_3O^{+}$$

A free radical was detected in the chitin molecule and was initially suspected of being involved in the coordination of uranyl ions to the nitrogen of the amine group (Tsezos, 1983). However, Muraleedharan and Venkobachar (1990) determined that the free radical detected in chitin-containing biosorbent material was not directly involved in binding of metals.

Chitin has been implicated in uranium adsorption by the filamentous fungus *Rhizopus arrhizus* (Tsezos, 1983).

Chitin's cation binding affinity order is:

$$Hg^{2+} > Pb^{2+} > Ag^{+}, Cu^{2+}, Ni^{2+} > Cd^{2+} > Co^{2+} > Fe^{2+} > Cr^{3+}$$

(Muzzarelli, 1977).

Chitosan:

Chitosan is produced by the deacetylation of chitin (Muzzarelli *et al*, 1980; McGahren *et al*, 1984) as depicted in figure 6.3, and is by nature a polycation when protonated. It is found in fungal cell walls (Grenier *et al*, 1991). Chitosan is capable of chelating trace transition metal ions from solutions of mixed ions at high ionic strength and its binding capacity is comparable to the artificial chelating resin Dowex A-1 (Muzzarelli, 1977; Muzzarelli, *et al* 1980). Chitin itself may bind heavy metal cations, but to a lesser degree than chitosan (Muzzarelli, 1977; Muzzarelli, *et al* 1980).

Chitosan's cation binding affinity order is:

$$Cd^{2+}, Cu^{2+} > Zn^{2+} > Co^{2+} > Fe^{2+} > Mn^{2+} > Ca^{2+}$$

(Muzzarelli, 1977).

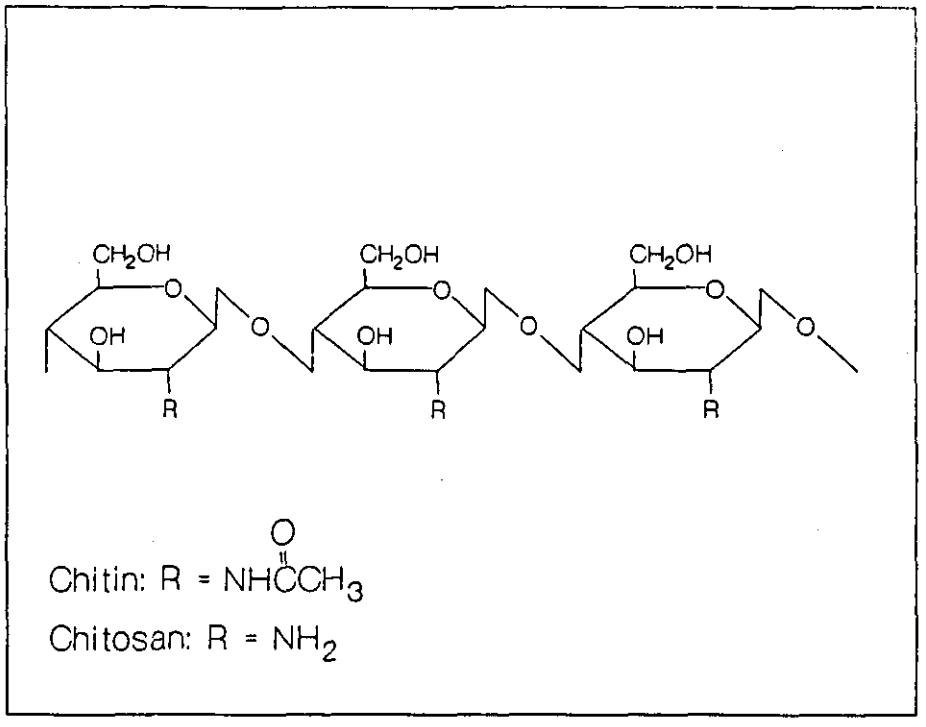


Figure 6.3: The central structural motif of chitin and chitosan.

Chitosan is selective for transition metals and does not accumulate large amounts of alkali or alkaline-earth metals (Muzzarelli, 1977). The nitrogen electrons present in the amino and substituted amino groups can form coordinate bonds with transition metal ions, particularly chitosan which has large numbers of free amino groups. Chitosan is also a polybase and therefore can form salts with transition metal oxo-anions (Muzzarelli, 1977). Cation accumulation is dependent on polymer grain size, temperature of reaction, degree of agitation (e.g. by stirring), ion oxidation state (ferric ions must be reduced to be removed, while ferrous ions are eluted easily), presence of competing ions, and pH (Muzzarelli, 1977). There may be more than one binding site for different metals as mixed metal solutions may show non-stoichiometric binding (Muzzarelli, 1977). Chitosan may

accumulate 10 times more metal than chitin, and was as good as, if not better than, any of a large range of natural and synthetic polymers at accumulating mercury cations (Muzzarelli, 1977). Chitosan membranes form chelates with Cu^{2+} cations at pH 4 - 5, which involve one or two nitrogen atoms. At higher pH the coordination number increases as the copper is coordinated to more nitrogen ligands of the amino groups and above pH 8, hydroxyl groups as well (Muzzarelli, 1980).

(f) Capsular polysaccharides:

Yeasts, like many other microorganisms, synthesize extracellular polysaccharides which give rise to capsules (Norberg and Enfors, 1982; Norberg and Rydin, 1984; Zosin *et al*, 1983). In the case of yeasts this includes phosphomannans. There is,however,little or no information on the production of these polysaccharide capsules by *S. cerevisiae* (Phaff, 1971).

6.1.5 The Bilayered Model of the Yeast Cell Wall:

The physical structure of the cell wall of *S. cerevisiae* is anisotropic. A model for the cell wall with an uneven distribution of the polymer constituents has been proposed to account for this fact (Hunter and Asenjo, 1988).

In this model the glucans (of assorted ß(1-3) and ß(1-6) linkage, which account for a large percentage of the cell wall) are found in two forms. The alkali-insoluble form consists of ß(1-3) glucan of approximately 1 500 dextrose polymer units (DP) with 3 - 6% ß(1-6) branch points. It is aggregated into microfibrils located towards the inner surface of the cell. The alkali-soluble fraction is similar, except that is has a greater percentage of ß(1-6) bonds and is linked

to small quantities of mannan oligosaccharide, which implies that it may be linked to the mannan-protein layer. The glucan fibril layer (with its associated chitin) is the most rigid and it serves to prevent osmotic lysis or mechanical breakage of the cell (Hunter and Asenjo, 1988). The outer layer consists of mannan polymers linked to proteins via asparagine residues. This matrix is cross-linked by disulphide bonds and intrachain hydrogen bonding (Phaff, 1971, Hunter and Asenjo, 1988). This layer filters the environment, preventing enzymes from attacking the inner glucan layer, while permitting passage of metabolites and other small molecules (Hunter and Asenjo, 1988). Being the outermost layer, it will also be that most exposed to chemical attack.

6.2 PREPARATION OF YEAST CELL WALLS

6.2.1 Introduction:

In order to study metal binding by isolated cell walls it is first necessary to separate the wall from the cell contents, and this can only occur by the formation of lesions in the encapsulating cell wall. These lesions may be produced by chemical or mechanical means. The cell walls isolated by the methods described here were then used in the following sections of this chapter in metal binding studies.

6.2.2 Autolysis of Yeast Cells:

(a) Methods:

Baker's yeast (*S. cerevisiae*) types I and II are known to autolyse at 37°C (Sigma Co). In this study yeast cells (1.5 $g.dm^{-3}$ dry mass as 5.0 $g.dm^{-3}$ wet mass) were centrifuged (1 000 x g for 15 min.) and the

pelleted cells re-suspended in 100 cm^3 of 50 $mmol.dm^{-3}$ KH_2PO_4 buffer (pH 7.4) containing 90 $mmol.dm^{-3}$ D-glucose, and maintained at 37°C for 0 - 22 hours. After this treatment the suspension was centrifuged (3 000 x g for 10 min.) to pellet whole cells and cell walls, and the protein content of the supernatant was determined by the Folin-Lowry method (Lowry *et al*, 1951) as an indication of protein release during cell lysis.

The supernatant was also assayed for general biological oxidation enzyme activity *via* oxidation of the substrate 2,6-dichlorophenol-indophenol (Plummer, 1978). The ability of the supernatant to oxidize 2,6-dichlorophenolindophenol was indicative of not only the amount of general protein released (a large number of different and specifically intracellular proteins are required to oxidize glucose), but also of their condition (reduced enzyme activity can be related to general protein denaturation and degradation).

(b) Results:

Autolysis of yeasts occurred to a limited extent only (figure 6.4). This indicates that the cell walls studied here were unlikely to have been damaged by autolytic enzymes to any large extent during cell wall isolation.

6.2.3 Mechanical Disruption of Yeast Cell Walls:

(a) Methods:

S. cerevisiae cell walls may be ruptured by use of high-pressure homogenizers (More *et al*, 1990), while many microorganisms may be disrupted by ultrasonication (Hughes *et al*, 1971). These methods and a number of alternatives were attempted in the present study (see table

6.2). All disruptions were completed at 4 - 10°C using ice and liquid nitrogen to minimize the effects of autolytic enzymes.

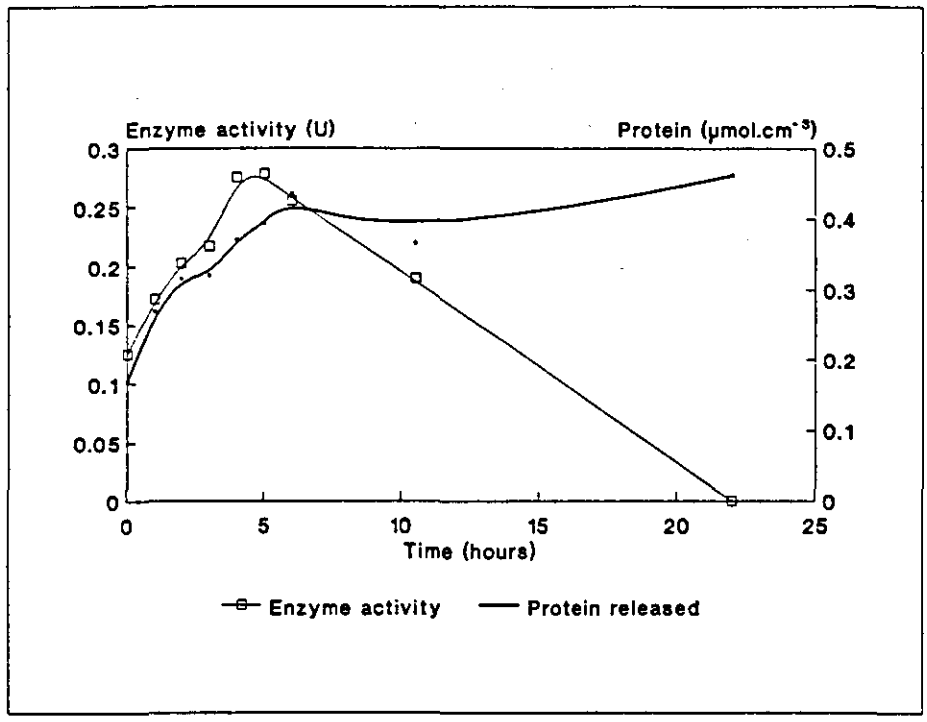


Figure 6.4: Autolysis of yeast cells at 37°C detected by protein release and enzyme activity. Normally intracellular enzyme activity in the cell-free supernatant peaks when extracellular protein concentration nears it maximum, this is assumed to be an indication of cell autolysis.

(b) Results:

Yeast cells proved to be extremely difficult to lyse, even by methods that were effective for other microorganisms. Upon lysis the cell walls could be seen as dark "ghosts" under phase-contrast microscopy (figure 6.5).

A number of physical disruption techniques were subsequently assessed for their ability to disrupt *S. cerevisiae* cells, the results of which are presented in table 6.2.

Differential centrifugation failed to separate cell wall "ghosts" from intact cells even at dilute cell suspension concentrations (1 $x10^{-8}$ $cells.cm^{-3}$) and low speeds (250 x g) and therefore only cell wall preparations from techniques that were more than 95% efficient could be used. As a result only the preparations from cells lysed by method number (8) in table 6.2 could be used; these preparations were supplied by the Department of Microbiology and Biochemistry, Orange Free State University, RSA. All ultrasonic and homogenisation processes were applied discontinuously to limit heat build up.

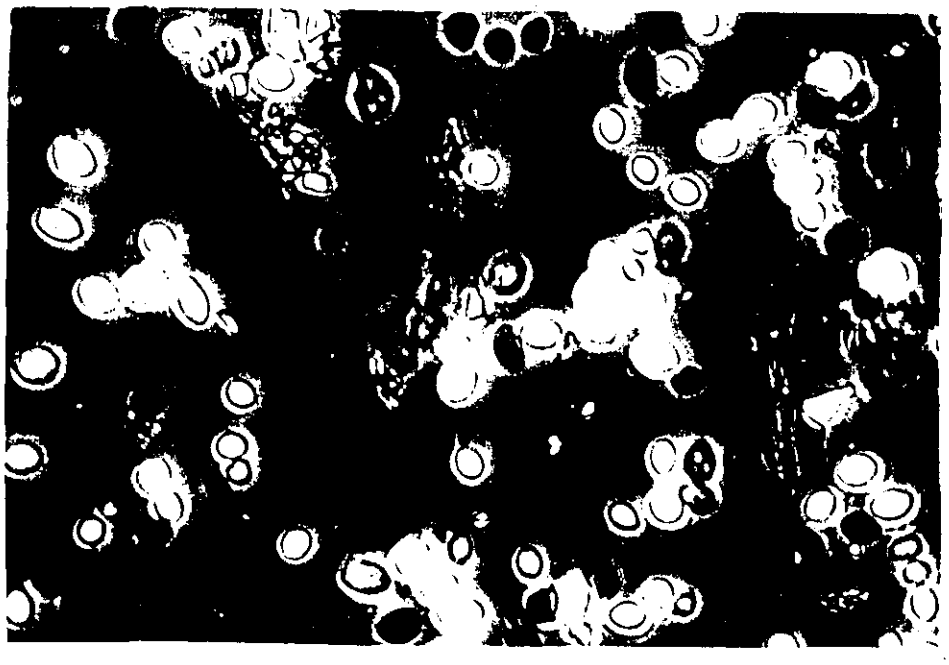


Figure 6.5: Lysis of yeast cells as seen in phase contrast microscopy (intact cells can be seen as the bright bodies and the lysed cells seen as the dark "ghosts").

TABLE 6.2: The efficiency of various techniques to lyse *S. cerevisiae* cells.

Lysis technique	% Lysis
1) Ultrasonication (30 min total)	1
2) Ultrasonication (30 min total) plus glass powder.	10
3) Homogeniser (Janke & Kunkel Ultra-turrax, probe type, 30 min total including glass powder).	37 - 43
4) Vortex-mixer with glass powder (12 min total).	2
5) Homogeniser (Waring commercial blender) with glass powder.	2
6) Grinding, freezing and sonication then Yeda press x3.	10 - 15
7) Ultrasonication and Yeda press x3.	5 - 10
8) Braun homogeniser, with glass beads.	95
9) French press cell (Aminco) at 9 tonnes pressure (3 passes).	30

6.3 BINDING OF METALS TO YEAST CELL WALLS

6.3.1 INTRODUCTION

Determining the role of isolated cell walls in metal accumulation not only gives a clearer view of metal binding by the cell wall but also, by simple deduction, places the accumulation properties of the remainder of the cell into perspective.

6.3.2 MATERIALS AND METHODS

Freeze-dried yeast cell walls (courtesy of Prof. B. Prior and Dr P. van Zyl) were resuspended in 5 mmol.dm^{-3} PIPES buffer and centrifuged

at 4°C at 3 000 x g for 10 min. and the supernatant discarded. The pellet was then incubated in buffer at room temperature for 6 hours and then freeze-dried.

Freeze-dried yeast cell walls (1 mg) were washed with ultra-pure water, pelleted by centrifugation (3 000 x g for 10 min.), and incubated with a copper solution (2 cm^3 of 1 000 - 5 000 $\mu mol.dm^{-3}$ copper (II) chloride salt in 5 $mmol.dm^{-3}$ PIPES buffer, pH 6.2) for 10 minutes at 22°C with frequent vigorous shaking using a vortex mixer. The suspension was then filtered using 0.45 μm pore size Millipore filters and the cell walls on the filter washed with 10 cm^3 of buffer. The retained cell walls and the filtrate were both assayed for metal content by the method of Norris and Kelly (1977) (see chapter 2).

6.3.3 RESULTS

The endogenous metal content of the yeast cell walls in the present study was (in $\mu mol.mg^{-1}$):

Ca^{2+}, 0.005; Na^{2+}, 0.0012; Cu^{2+}, Co^{2+}, Cd^{2+} < 0.0001.

The binding of Cu^{2+} by yeast cell walls at various copper concentrations is depicted in figure 6.6. Copper accumulation was found to be dependent on the initial ambient copper concentration.

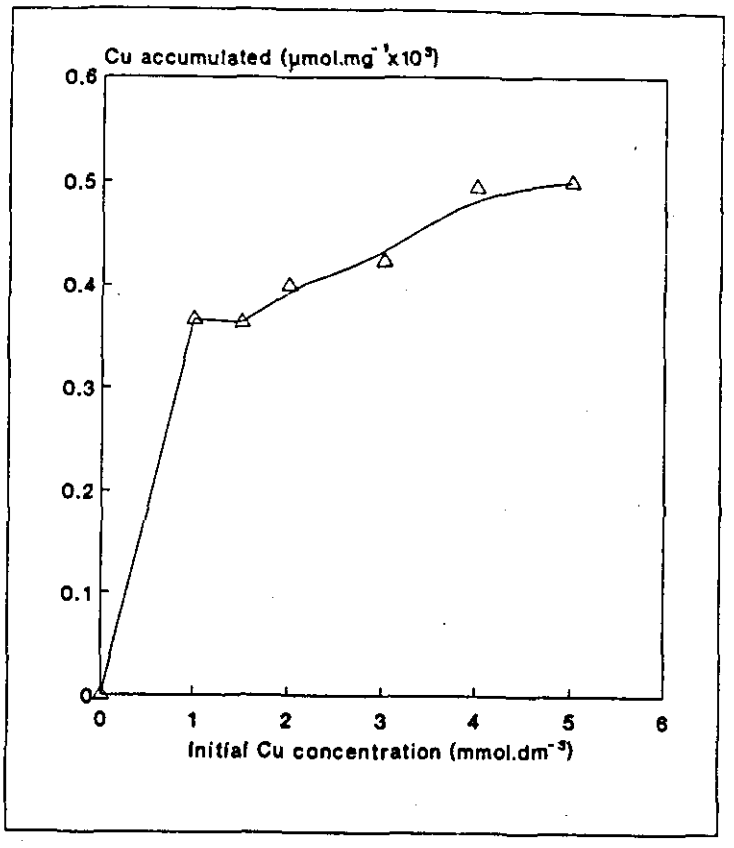


Figure 6.6: Binding of copper by *S. cerevisiae* cell walls as a function of initial copper concentration.

Isotherms and Scatchard Plots:

An isotherm plot may be used to graphically represent metal ion accumulation by a biosorbent, in this case as a function of moles of metal bound per mass cell wall against the residual metal concentration in solution (in moles per volume). The advantage of an isothermal plot compared to the type of plot represented in figure 6.6 is that an isothermal plot is independent of the volume of metal salt solution reacted with the cell walls. The relationship between metal accumulation and residual metal concentration is most often hyperbolic (Volesky, 1987). The maximum biosorption limit of the material is important in that this is a measure of the ability of the material to accumulate metals from solutions of high metal concentrations. Another parameter that must be noted is the steepness of the isotherm

as it leaves the origin, which indicates the ability of the biosorbent to accumulate metals from dilute solutions. This is a measure of the affinity between the ligands on the biosorbent and the metal ion species in question, which can be seen more clearly when the data from a isotherm curve is replotted using a Scatchard transformation (Scatchard, 1949). The Scatchard plot allows for determination of the affinity constant between the cation and the ligand (from the slope of the graph) and the number of binding sites (at the intercept with the abscissa). Isotherms and Scatchard plots of metal cation binding to the isolated cell walls of *S. cerevisiae* can be seen in figures 6.7 and 6.8 respectively, while the metal binding parameters derived from figure 6.8 are tabulated in table 6.3. Yeast cell walls bound copper in greater quantities than cadmium which in turn was bound in greater quantities than cobalt cations.

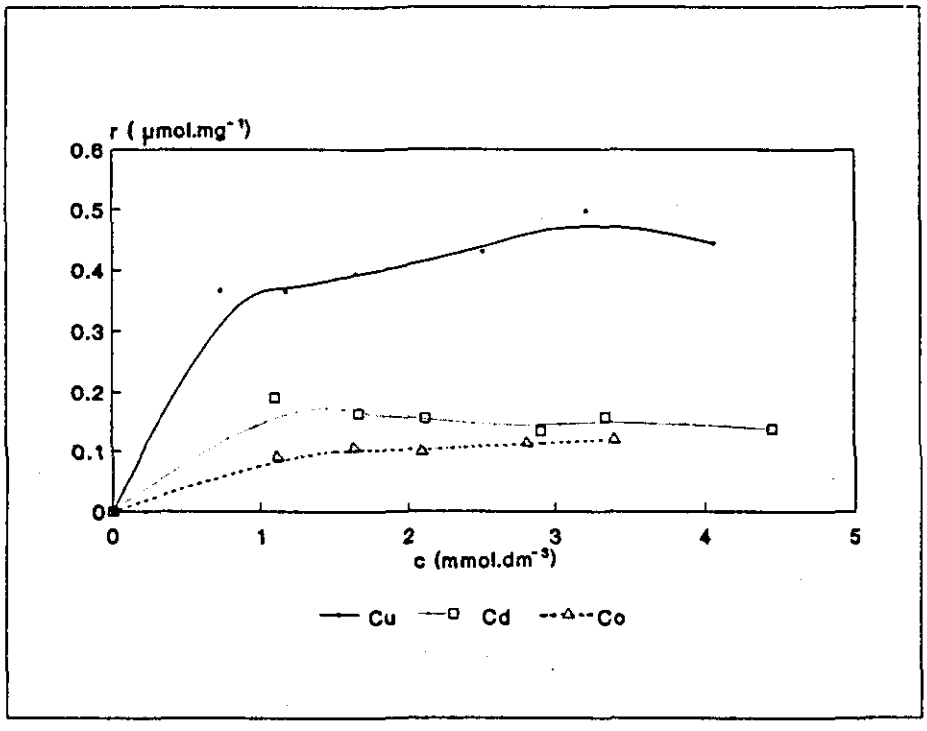


Figure 6.7: Isotherm of metal accumulation by *S. cerevisiae* cell walls. (r) is µmoles of metal bound per mg cell wall, (c) is the concentration of unbound metal.

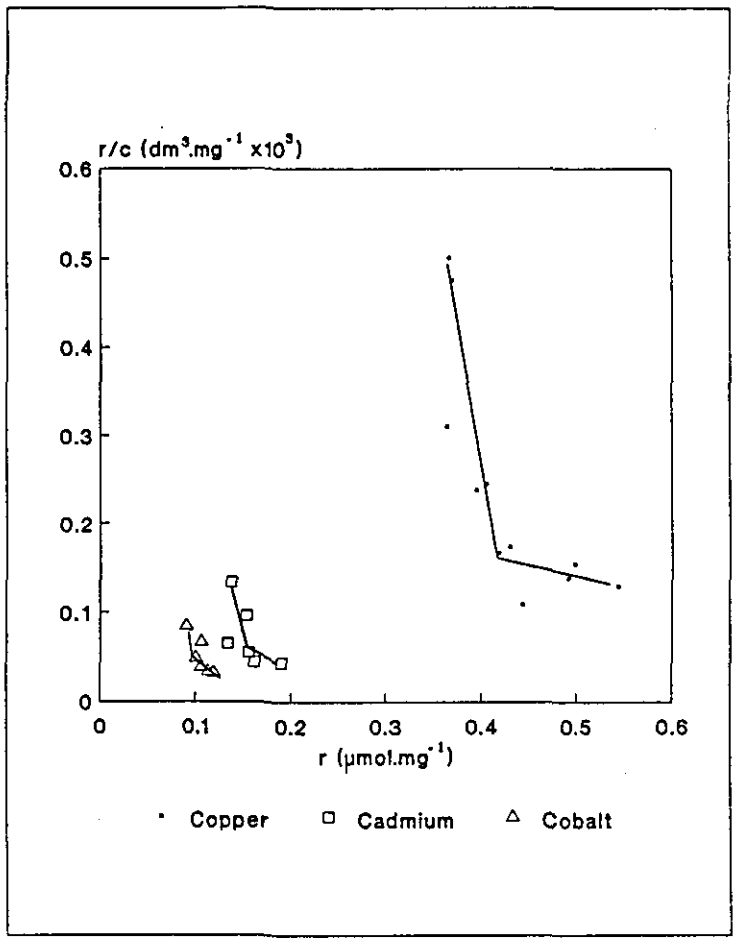


Figure 6.8: Scatchard plot of metal cation binding by the cell wall of *S. cerevisiae*. (r) is µmoles of metal bound per mg cell wall, (c) is the concentration of unbound metal.

The values in table 6.3 are approximate and represent extrapolations based on the data for the lower initial metal concentrations used.

TABLE 6.3: Maximum copper binding sites on yeast cell walls and their association constants.

Metal	Maximum binding sites ($\mu mol.mg^{-1}$ wall dry mass)	K_a ($dm^3.mol^{-1}$ $x10^{-3}$)
Cu^{2+}	0.40	4.8
Co^{2+}	0.11	3.8
Cd^{2+}	0.17	3.7

6.3.4 DISCUSSION

The Scatchard plot in the present study was non-linear. Non-linear Scatchard plots are an indication of non-homogeneity of binding sites (Tobin *et al*, 1990). A biphasic plot has two possible interpretations, indicating either multiple classes of non-interacting sites, or as interacting sites with positive or negative co-operation (Doyle *et al*, 1980). Tobin *et al* (1990) used Scatchard analysis to determine metal binding by denatured *Rhizopus arrhizus* and found more than one type of binding site. This makes it difficult to assign an overall affinity constant or maximum number of binding sites as each sub-set of sites has its own affinity constant. Even linear Scatchard plots only represent the average binding affinity of the cell wall and it is not necessarily the case that these sites are homogeneous.

Rothstein and Hayes (1956) obtained biphasic Scatchard plots of Mn^{2+} binding to the yeast surface, the lower slope apparently due to additional binding sites with a lower affinity for the cation, but the exact values of the constants cannot be readily determined as it is difficult to separate the two slopes. They found that the binding was rapid and reversible, obeying the mass law equation. Bivalent cations were found to bind more strongly than monovalent cations. Similar to the present study, they did not attempt to maintain constant ionic strength, as any added ions would compete with the heavy metal ions for binding sites. The order of binding was: $UO_2^{2+} >>> Zn^{2+}$, $Co^{2+} > Ca^{2+}$, Cu^{2+}, $Hg^{2+} > Na^{+}$, K^{+}. In the present study, however, Cu^{2+} was found to bind in greater quantities than Co^{2+}.

In conclusion, isolated cell walls of *S. cerevisiae* bind Cu^{2+}, Cd^{2+}, and Co^{2+} in different quantities. Binding sites are apparently common for all metals, but some binding sites preferentially bind particular cations (Tobin *et al*, 1988, 1990). There appear to be a range of binding sites on the yeast cell walls with various affinities for these metals.

6.4 CHEMICAL MODIFICATIONS OF YEAST CELL WALLS

6.4.1 INTRODUCTION

Chemical modification of cell walls prior to metal binding reactions has been used by numerous researchers to determine which of the chemical groups located on microbial cell walls are involved in binding metal cations. The application of these methods to the isolated cell walls of *S. cerevisiae* could reveal not only which types of ligands are available for metal cation binding (and by deduction which macromolecular cell wall components are involved), but also whether inexpensive chemical reactions could be used to enhance metal biosorption.

6.4.2 MATERIALS AND METHODS

(A) AMINO GROUPS

(a) *S*-Acetylmercaptosuccinic anhydride addition to amino groups (Beveridge and Murray, 1980). Amino groups were chemically modified by the addition of *S*-acetylmercaptosuccinic anhydride so that both a carboxyl and a sulphydryl group were introduced to the cell wall,

while the amino group was neutralized (figure 6.9). The *S*-acetyl-mercaptosuccinic anhydride was dissolved in ethanol to yield a 20 $mmol.dm^{-3}$ solution. A 5 cm^3 aliquot of this solution was added to a suspension of yeast cell walls (50 mg dry mass of walls in 45 cm^3 of deionized water) to produce a final anhydride concentration of 2 $mmol.dm^{-3}$. The reaction was carried out under nitrogen gas at pH of 6.8 with constant stirring for 6 h at 22°C. At this time the walls were pelleted by centrifugation, washed thrice with 50 cm^3 volumes of deionized water with centrifugation, and dialized against 6 dm^3 of deionized water for 12 hours at 4°C.

(b) Acetic anhydride addition to amino groups (Doyle *et al.* 1980). Amino-acetylation was performed by the addition of three 1.0 $x10^{-4}$ dm^3 volumes of acetic anhydride to a stirred wall suspension (10 cm^3 of 5 $mg.cm^{-3}$) in a 1/2 saturated sodium acetate solution. The three anhydride volumes were added at 12 minute intervals. The acetylated wall was centrifuged, washed with cold water, and resuspended in 1.0 $mol.dm^{-3}$ hydroxylamine (pH 8.0) to remove O-acetyl groups. The amino acetylated cell wall was finally washed with water and freeze-dried. The amino groups were thereby neutralized (figure 6.9).

(c) Succination of the amino groups (Doyle *et al.* 1980). Cell walls were succinylated by adding solid succinic anhydride (total of 72 mg) to 10 cm^3 of a cell wall suspension (5 $mg.cm^{-3}$) in 1.0 $mol.dm^{-3}$ sodium carbonate. Succinic anhydride was added in 9 mg quantities at 10 minute intervals. The modified walls were finally treated with hydroxylamine (as described above for acetylated walls), washed, and freeze-dried. The amino group was neutralized and a carboxyl group was added (figure 6.9).

(B) AMINO AND HYDROXYL GROUPS

Acetate addition to amino and hydroxyl groups (Beveridge and Murray, 1980). Fifty milligrams of cell walls was treated with 50 cm^3 of aqueous 0.05 $mol.dm^{-3}$ sodium iodoacetate, maintained at pH 8.0 for 6 hrs at 22°C, then washed and dialized as outlined above. This reagent typically attaches to amino groups at low or neutral pH (figure 6.9), but may also bind to phenolic or hydroxyl groups at higher pH.

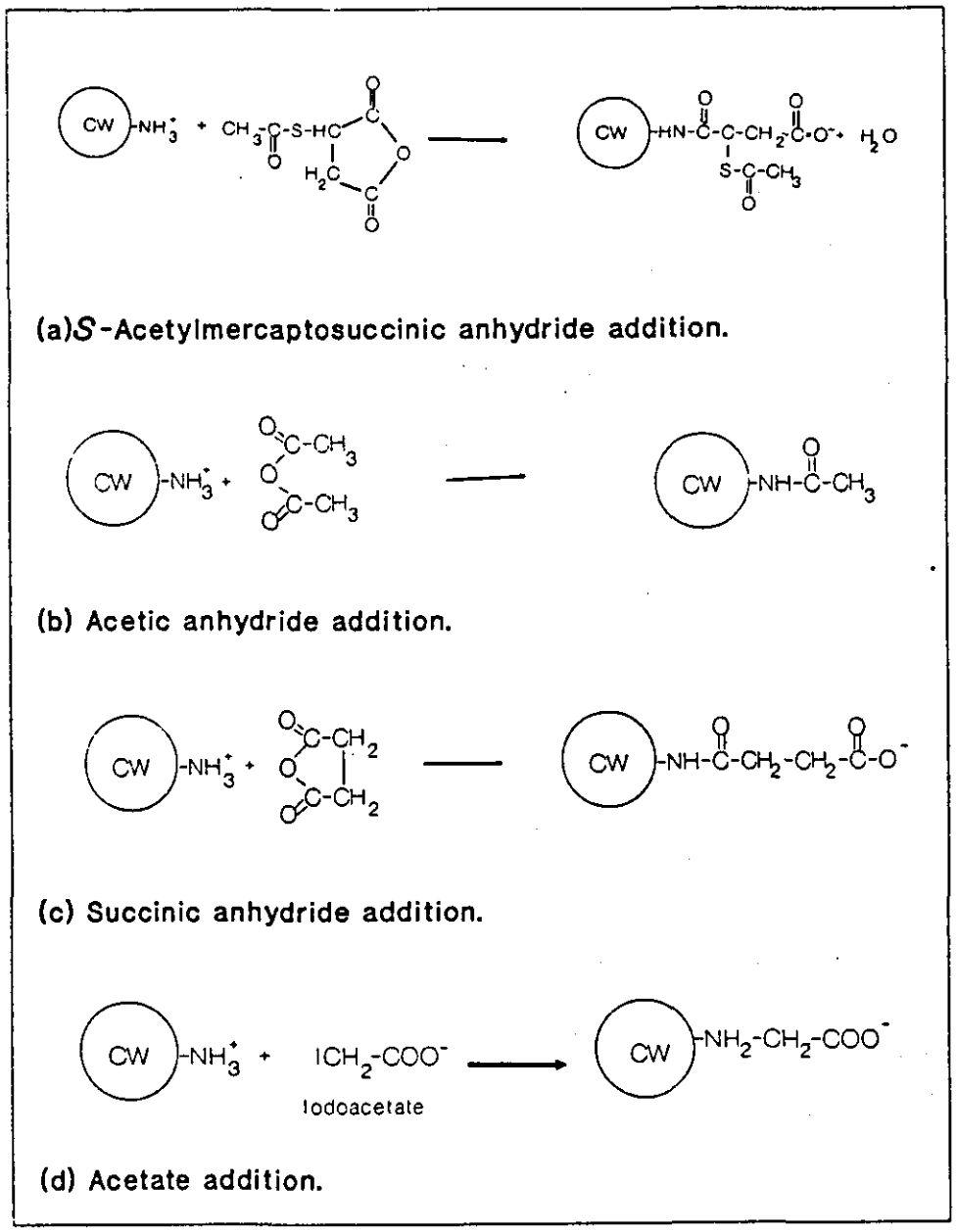


Figure 6.9: Chemical modifications of amino groups.

(C) CARBOXYL GROUPS

(a) Esterification of carboxyl groups (Beveridge and Murray, 1980). Three chemical ligands of different electrochemical charge were attached by means of the carbodiimide reaction: glycine ethyl ester neutralized the carboxyl charges of the wall, glycinamide made the walls modestly electropositive, and ethylene diamine made them distinctly electropositive (figure 6.10). In each case the respective ligands were added to a 50 cm^3 aqueous suspension of 50 mg native yeast cell walls to give a final concentration of 0.5 $mol.dm^{-3}$, and sufficient 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride was included to make the carbodiimide concentration 0.2 $mol.dm^{-3}$. The reaction mixture was maintained at 22°C and continuously stirred for 6 hours at pH 4.75, and subsequently washed and dialyzed as previously described.

(b) Methylation of carboxyl groups (Tobin *et al.*, 1990). The methyl iodide treatment results principally in esterification of biomass carboxyl groups, thereby neutralizing them (figure 6.10). Methyl iodide is a strong alkylating agent and may also result in the displacement of H-atoms on $-NH_2$ and -OH groups. The expected effect is to inhibit carboxyl groups from participating in the uptake process. In this method 50 mg of freeze-dried native yeast cell walls were stirred with 20 cm^3 of methyl iodide for 4 hours at 22°C, and subsequently washed and dialyzed as previously described.

(D) PHOSPHATE GROUPS

Ethylation of phosphate groups (Tobin *et al.* 1990). Triethyl phosphite-nitromethane treatment results in esterification of the phosphate groups of orthophosphoric acid. Groups present in monoester

diester form are likely to be further esterified. It is expected, therefore, that a portion of the biomass phosphate groups will be excluded from metal uptake by this treatment. In this method 50 mg of freeze-dried native yeast cell walls were heated under reflux with 25 cm^3 of triethyl phosphite [$(C_2H_5O)_3P$] and 20 cm^3 of nitromethane (CH_3NO_2) for 6 hours, and subsequently washed and dialyzed as previously described.

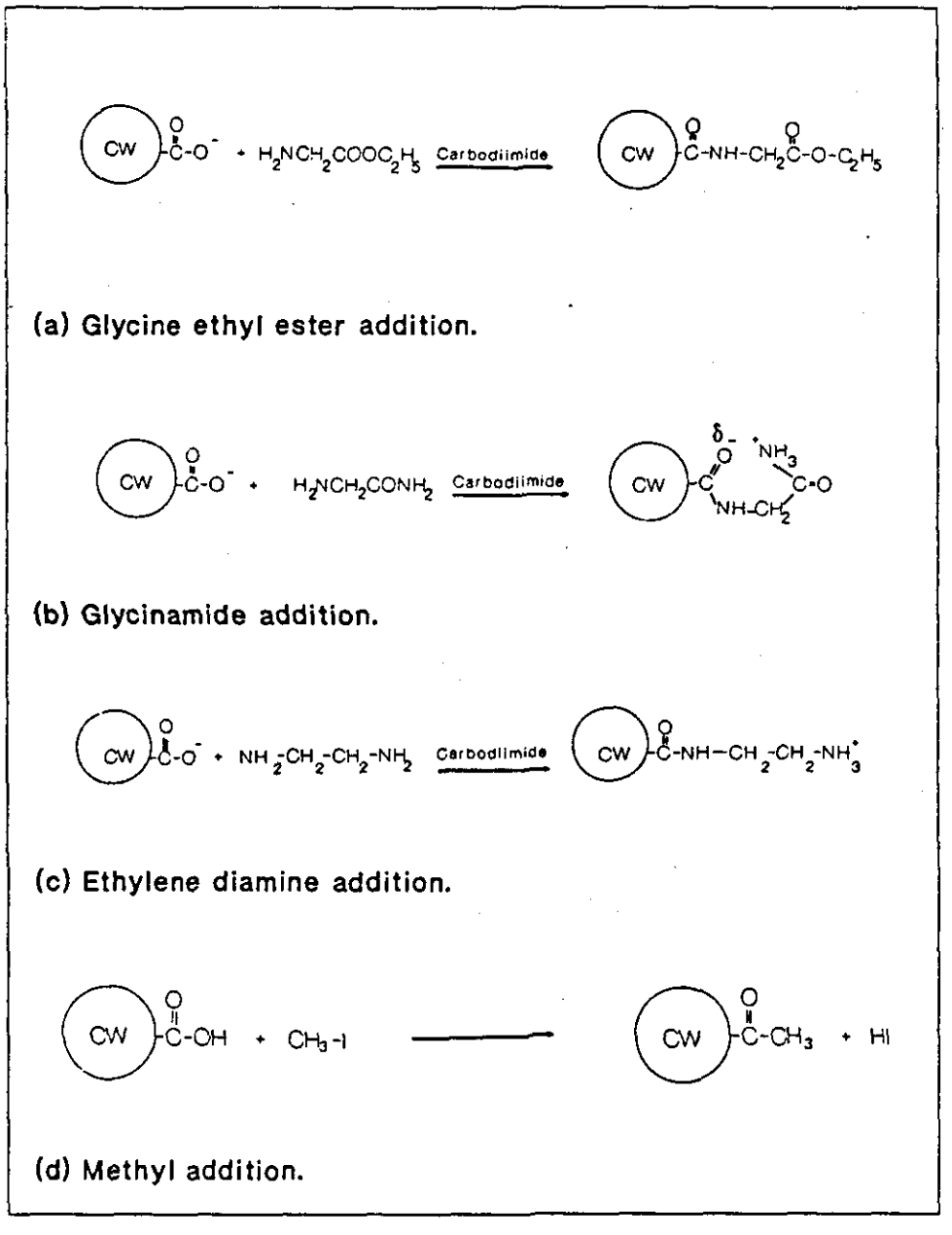


Figure 6.10: Chemical modifications of carboxyl groups.

6.4.3 RESULTS

The functional groups were modified under mild conditions, which reduced the possibility of unwanted alterations to the cell wall. More drastic chemical modifications could cause an overall decrease in metal uptake capacities (Tobin *et al*, 1990). Comparison of the infra-red spectra of chemically modified cell walls to those of native cell walls was intended to be used to determine the effectiveness of the various ligand chemical modification techniques. The spectra of the modified cell walls were similar to that of native cells (figure 6.11) and the peak shifts were difficult to interpret using this method without other spectroscopic techniques, as the peaks produced by the original and modified chemical groups tended to overlap to a large extent.

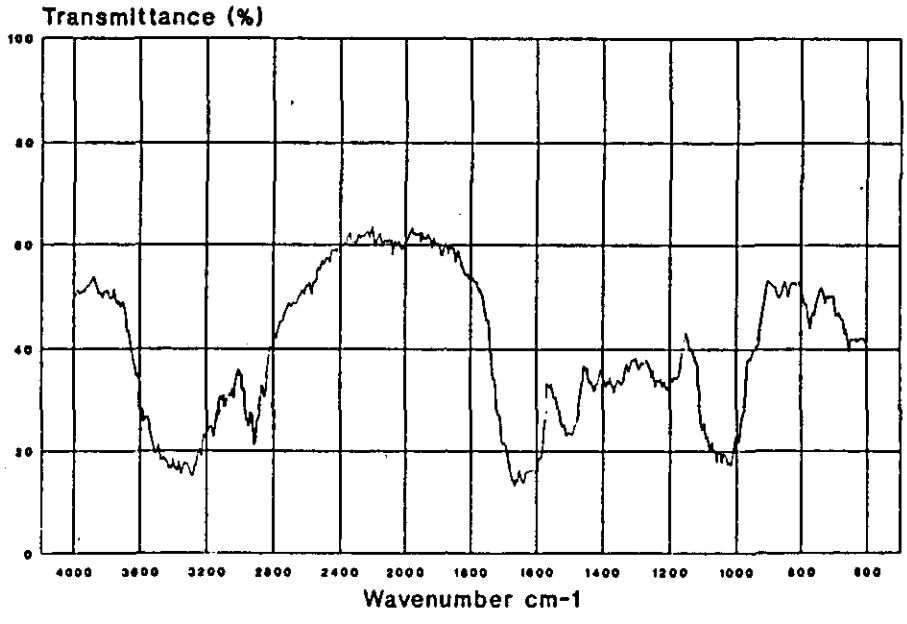


Figure 6.11: Infra-red spectrum of the yeast cell wall.

However, the various products of chemical modifications of yeast cell walls were different in their physical appearance to each other and to the native cells. Chemical modification of the phosphate groups yielded a product that had been drastically physically altered in appearance and was erratic in its capacity to bind Cu^{2+} due to being inhomogeneous (data not shown).

The effect of the chemical modifications of the yeast cell wall groups on copper binding can be seen in Scatchard plots (figures 6.12 and 6.13). The slopes of the Scatchard plots are biphasic or multiphasic, indicating that there are more than one type of Cu^{2+} binding site on the cell wall.

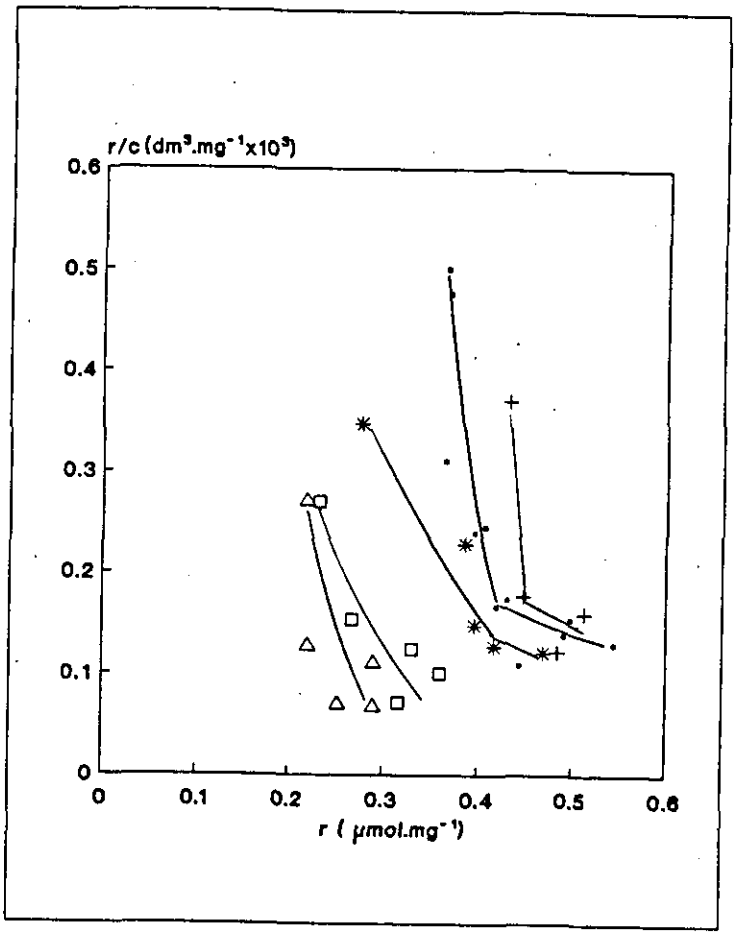


Figure 6.12: Scatchard plot of the accumulation of copper cations by chemically modified cell walls of *S. cerevisiae*, with modification of amino groups. • Native cell walls; + *S*-acetylmercaptosuccinic anhydride; * succinyl anhydride; □ acetyl anhydride; Δ iodoacetate.

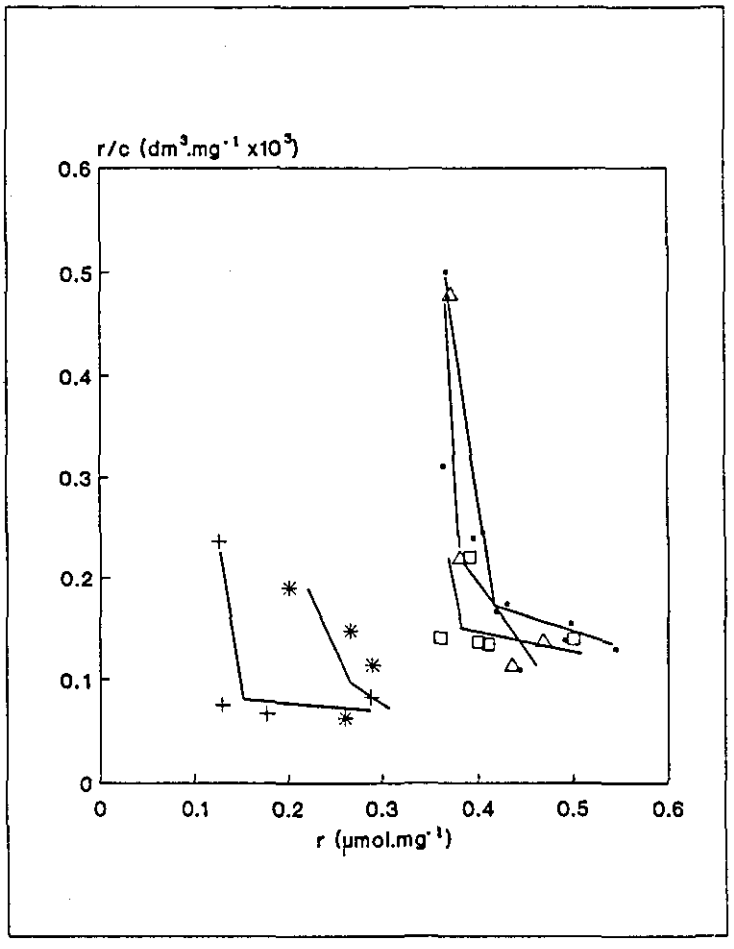


Figure 6.13: Scatchard plot of the binding of copper cations by chemically modified cell walls of *S. cerevisiae*, with modification of carboxyl groups. • Native cell walls; + glycinamide; * glycine ethyl ester; □ ethylenediamine; Δ methyl iodate.

6.4.4 DISCUSSION

The nature of the cation binding to cell wall ligands in various microbial species has been previously studied. The displacement of hydrogen ions during cation accumulation by algal cell walls was found to depend on the cation in question; Na^+ displaced none while Cu^{2+} displaced an average of 1.2 H^+ for each copper ion accumulated (Crist *et al*, 1981). The extent of proton displacement would of course depend

on part on the ambient pH, which would determine the protonation state of the cell wall ligands. The fact that Cu^{2+} binds to a greater degree than Na^{+} suggests that coordination bonds play an important role in cation binding, while the partial displacement of copper by sodium ions indicates that at least some ionic bonding is involved (Crist *et al*, 1981). A common seaweed, *Ascophylum nodosum*, was found to accumulate cobalt, and the major mechanism for this was suggested to be ion exchange on the alginate carboxyl groups (Kuyucak and Volesky, 1989).

The mechanism of binding of heavy metals by isolated *S. cerevisiae* cell walls has not been investigated by means of chemical modification previously. In the present study most chemical modifications to the yeast cell wall decreased copper accumulation. Modification of either carboxyl or amino groups reduced copper accumulation. Other studies involving chemical modification of carboxyl groups on *Bacillus subtilis* (a Gram positive bacterium) cell walls greatly reduced metal cation accumulation by the cell walls, whereas modification of the amino groups had little effect (Beveridge and Murray, 1980; Doyle *et al*, 1980).

There is a valid reason for this, which is integral to the study of biosorption. Of the divalent ions studied by Beveridge and Murray (1980), Cu^{2+} behaved differently from the other cations in that it bound preferentially to amino groups over carboxyl groups, and modification of amino groups by both *S*-acetylmercaptosuccinic anhydride and sodium iodoacetate reduced the Cu^{2+} binding capacity drastically. In the present study neutralization of amino groups with glycine ethyl ester decreased the binding of copper slightly, and

glycinamide even more so. The addition of ethylenediamine resulted in binding capacities similar to those of the native cell wall. The addition of glycinamide also results in the addition of an amino group, but this is probably involved in intramolecular binding with a polarized carbonyl group (see figure 6.10).

Modification of carboxyl groups of *B. subtilis* cell walls led to complete loss of affinity for monovalent cations while only a partial decrease in the binding of multivalent cations occurred (Beveridge and Murray, 1980), which suggests that non-ionic groups are to a large extent responsible for binding divalent cations.

The isolated cell walls of *B. subtilis* have also been chemically modified by Doyle *et al* (1980). The free carboxyl and amino groups were replaced by neutral, bulky, or negatively charged groups by various reactions. Where these reactions introduced positive charges into the cell wall there was a decrease in the numbers of metal binding sites, and usually a concomitant decrease in the apparent association constants for certain cations, i.e. Na^{+}, Ca^{2+}, Ni^{2+} and Zn^{2+}, metals which tend to prefer harder ligands.

High hydrogen ion concentration (i.e. low pH) reduces metal binding, suggesting that cations and protons compete for the same sites (Doyle *et al*, 1980; Marquis *et al*, 1976). In terms of cation binding to the yeast cell wall amino group it is of interest to speculate on which macromolecular components of the yeast cell wall contain amino groups. Protein content of the yeast cell wall was originally calculated from the nitrogen content of the cell wall (2.1%) by the formula 2.1% x (6.25) = 13.1% (Northcote and Horne, 1952; Korn and Northcote, 1960;

see table 6.1). From this figure must be deducted the nitrogen bound in chitin and chitosan (0.25%). Therefore the protein content of the cell wall would be approximately 12.85%. Since no other major nitrogen containing polymeric compound has been found in the yeast cell wall then nearly all the available nitrogen is associated with protein, chitin and chitosan. Any metal preferentially binding to amino groups must therefore be binding to these three polymers, and mostly to protein. As the copper in the present study was at least partially bound to amino groups, then it may be assumed that a significant percentage of the bound copper cations was probably bound to cell wall protein. During mercury accumulation by yeast cells the major fraction of mercury was tightly bound to the cell wall with protein being implicated in the binding (Murray and Kidby, 1975).

Sulphydryl groups have been shown to be involved in the binding of metals by *Chlorella vulgaris* (Darnall *et al* 1986). The addition of a sulphur diester in the present study (as *S*-acetylmercaptosuccinate) increased the accumulation of copper slightly even though it neutralized the amino groups to which copper may bind, demonstrating the importance of sulphur as a cation binding group.

The metal-ligand interaction is dependent on the three-dimensional arrangement of the ligands, and chemical modifications by their very nature disrupt this arrangement. While chemical modifications may yield some clues to the mechanism of metal binding, a more realistic evaluation may be possible through non-invasive techniques such as nuclear magnetic resonance spectroscopy which, although originally crude, are becoming extremely sophisticated.

6.5 ENZYMATIC MODIFICATION OF YEAST CELL WALLS

6.5.1 INTRODUCTION

The use of enzymes to specifically remove certain components from the yeast cell wall could yield information about the possible importance of these components in the binding of metals. This information could be obtained by observing how metal accumulation by the cell wall is modified by the removal of these components. This type of approach is relatively novel and may give more realistic descriptions of the relationship of the cell wall structure to biosorption of heavy metal cations than chemical extractions (see section 6.6) which are by nature less specific and chemically harsher. The aim of this section of the present research is therefore to observe *in situ* binding of copper cations by yeast cell walls deficient in specific polymers usually found in the cell wall.

6.5.2 MATERIALS AND METHODS

Enzymatic digestion of isolated yeast cell walls was carried out as described below. Digested cell walls were washed with ultra-pure water by centrifugation (3 000 x g for 10 min.). Protease, α-mannosidase and chitinase enzyme preparations were assayed for cross-specificity with the assay methods descibed as stated under the relevant enzymatic digestion process. Enzyme activity during assays was detected by colour formation. All enzymes used here were obtained from Sigma Co. and dialysed against ultra-pure water before use.

(a) Protease digestion of the cell wall

Pronase, the multiple exo- and endopeptidases of *Streptomyces griseus* K-1, possesses a very broad specificity for peptide sequences (Narahashi *et al*, 1968). Pronase (150 units) was used to digest 50 mg (dry mass) of freeze-dried native cell walls at 37°C in 0.05 mol.dm^{-3} Tris buffer (pH 7.5) with shaking for 6 hours. Tris (base) was supplied by Serva, Heidelburg (A.R. grade).

Protease assay

Protease activity was determined by a method similar to that of Boethling (1975). A 1 cm^3 aliquot of a suitable enzyme dilution was pre-warmed with an equal volume of distilled water for 5 minutes at 37°C. A 1 cm^3 aliquot of casein solution (10 mg.cm^{-3} casein in 0.05 mol.dm^{-3} Tris buffer, pH 7.5; initially dissolved at 70°C), was pre-warmed separately and added to the dilute enzyme solution. After exactly 15 minutes of incubation at 37°C the reaction was terminated by addition of 2 cm^3 of 10% trichloroacetic acid (BDH, England). The reaction tube was immediately placed in a 0°C water bath for a duration of at least one hour to allow the precipitation of insoluble denatured protein to occur.

The solution was then filtered through Whatman No. 1 filter paper and the filtrate was assayed for peptides and free amino acids by the Folin-Lowry method at 500 nm. Controls of each tube were treated in the same manner except that the 10% trichloroacetic acid was added prior to addition of the casein solution.

(b) Chitinase digestion of the cell wall

Chitinase (1 unit) was used to digest 50 mg (dry mass) of freeze-dried native cell walls at 25°C in PIPES buffer (0.1 mol.dm^{-3}, pH 6.1, adjusted with tetramethylammonium hydroxide) with shaking for 6 hours.

Chitinase assay

The assay was carried out in PIPES buffer (0.1 mol.dm^{-3}, pH 6.1) at 25°C for 6 hours using chitin azure (2 mg.cm^{-3}) as the substrate. This chitinase substrate is chitin dyed with Remazol Brilliant Violet 5R, according to the method of Hackman and Goldberg (1964), and supplied by Sigma Co.

(c) Laminarinase digestion of the cell wall

Laminarinase (2.5 units) was used to digest 50 mg of cell wall (dry mass) at 37°C at pH 5.0 in 0.1 mol.dm^{-3} acetate buffer with shaking for 6 hours. The Laminarinase enzyme also contains cellulase and α-amylase activities (Sigma Co.).

(d) Mannosidase digestion of the cell wall

Mannosidase (50 units) was used to digest 50 mg of cell wall (dry mass) at 25°C at pH 4.5 in 0.1 mol.dm^{-3} acetate buffer with shaking for 6 hours.

Mannosidase assay

α-Mannosidase from jack beans was incubated with substrate (p-Nitrophenyl-α-D-mannose (Sigma Co.)) at pH 4.5 in 0.1 mol.dm^{-3} acetate buffer at 25°C for 6 hours.

(e) ß-Glucuronidase digestion of the cell wall

ß-Glucuronidase (type H-1, partially purified from *Helix pomotia*, 13.9 $x10^3$ units) was used to digest 50 mg of cell wall (dry mass) at 37°C in 0.1 $mol.dm^{-3}$ sodium acetate buffer (pH 5.0) with shaking for 6 hours. The enzyme preparation may contain acid phosphatase activity depending on the original suppliers (Sigma Co.)

(f) Quantification of protein and carbohydrate content of the cell wall:

Samples of the cell wall components were hydrolysed before chemical analysis. Chitin (1 mg) was digested in 1 cm^3 of 4 N HCl at 100°C for 4 hours (Catley, 1988), while all other samples were hydrolysed for 6 hours at 100°C with 2 N HCl. $Mannan_A$ was first dialysed against 0.1 $mol.dm^{-3}$ EDTA in ultra-pure water and then against ultra-pure water (4hours for 4°C each dialysis) to remove complexed copper prior to acid hydrolysis.

Protein assay

The Folin-Lowry assay was used to determine protein contents of cell wall acid digest samples (Lowry *et al*, 1951; Clark and Switzer, 1977). Standards are presented in Appendix 3.

Carbohydrate assay

Nelsons test for reducing sugars (Clark and Switzer, 1977) was utilized to determine the amount carbohydrate present in each of the cell wall acid digest samples (standards are presented in Appendix 3).

(g) Copper binding assays:

The relative Cu^{2+} binding capacity of the enzymatically digested cell walls was determined. PIPES, a hydrogen ion buffer that does not chelate copper (Good *et al*, 1966), was used for copper binding studies. The methodology is described in section 6.3.2.

6.5.3 RESULTS

The cross-specificity of the enzymes used for cell wall degradation was determined (table 6.4). There was in fact little cross-specificity detected. This implies that in most cases each enzyme preparation specifically removed a single type of macromolecular component from the isolated yeast cell walls.

TABLE 6.4: Enzyme preparation specificity for cell wall components using standard substrates.

Enzyme	Enzyme activity		
	Protease	α-mannosidase	chitinase
Pronase	+++	-	-
Chitinase	+	-	++
Laminarinase	-	-	-
Mannosidase	-	+++	-
ß-Glucuronidase	-	-	-

The accumulation of Cu^{2+} by the partially degraded cell walls can be seen in table 6.5. The copper binding is only related to the protein-to-carbohydrate ratio to a limited extent, although the preparations with the lower protein-to-carbohydrate ratios tend to exhibit decreased Cu^{2+} binding. Although where the majority of the protein has been removed from the cell wall the least Cu^{2+} binding occurred (table 6.5), this also happened to be the cell wall preparation with the lowest percentage yield of product after enzymatic degradation (table 6.5). Indeed, from the graphic plot of this data (figure 6.14) it can be seen that there is a correlation between Cu^{2+} accumulation and the yield of cell wall material remaining after enzymatic degradation, suggesting a link between cell wall integrity and copper accumulation.

TABLE 6.5: Copper cation accumulation by cell wall preparations modified by enzyme action

Enzyme treatment	Protein/ carbohydrate ratio	Yield %	Copper binding ($\mu mol.mg^{-1}$)
Native	0.159	-	0.44
Pronase	0.050	20.2	0.31
Chitinase	0.159	51.2	0.43
Laminarinase	0.159	50.0	0.46
Mannosidase	0.353	72.2	0.51
ß-Glucuronidase	0.419	42.0	0.44

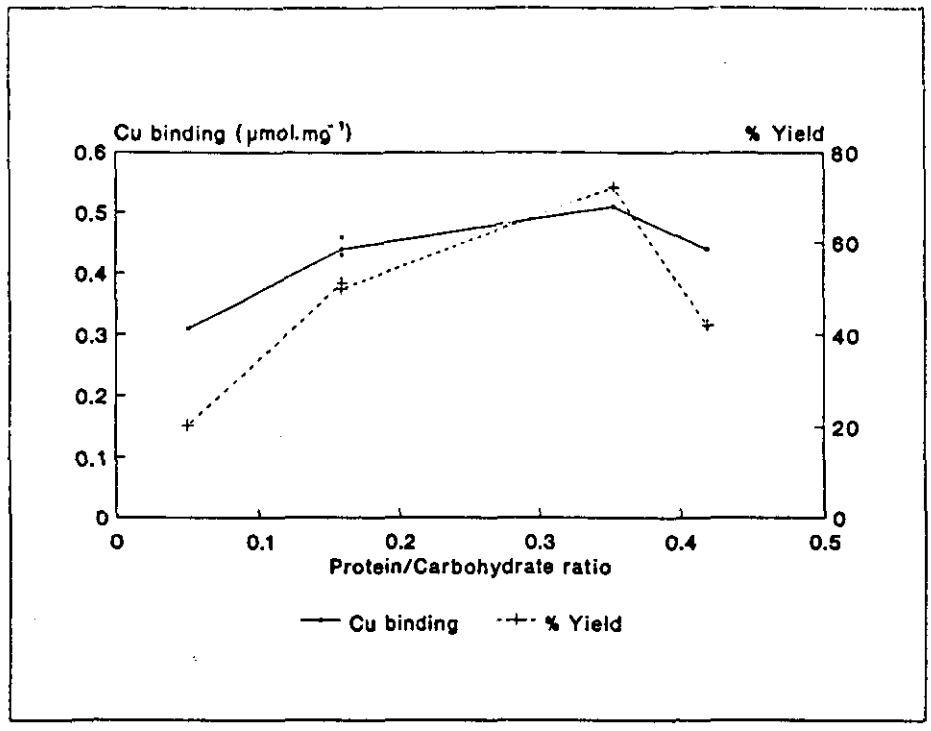


Figure 6.14: Relationship of protein/carbohydrate ratio to copper cation binding by cell wall preparations that had been previously modified by enzyme action.

6.5.4 DISCUSSION

The cell wall is anisotropic, with the outer layer (mannan-protein) contributing less than the inner layer to the supportive structure, but necessary for cell protection and control of nutrition and toxins passing into the cell (Catley, 1988).

Based upon a two layer model of the yeast cell wall (first observed by Northcote and Horne, 1952), studies on the process of enzymatic lysis of the yeast cell wall (Hunter and Asenjo, 1988; Liu *et al*, 1988) developed a paradigm that when applied to the present results is very enlightening. Protease attacks the outer layer of the cell wall and

may lead to its complete removal. However the mannan-protein layer is only removed to a limited extent by treatment with mannanase (Hunter and Asenjo, 1988). Enzymatic lysis experiment suggested that mannan digestion did not noticeably modify the accumulation of copper, yet protein digestion did. The yields of reaction of the present enzymatic cell wall degradation experiments suggests that the protease digestion decreased the yield of cell wall far more than did the mannosidase digestion. What may be concluded is that the mannan-protein layer is probably almost completely removed by protease activity, while mannanase activity only removes some of the mannan and not the entire layer. The protein then appears to be the "glue" which affixes the two wall layers together. If this is true then it is apparent that the outer layer of the yeast cell wall is more important in copper accumulation than is the inner layer.

Other researchers have also remarked on the importance of the outer layer in metal cation binding. Lyons and Hough (1970) noted that removal of the manno-protein layer with its phosphodiester links by proteases decreased the cation binding capacity of the isolated cell wall by 50 %.

However, experiments by Muraleedharan and Venkobachar (1990) suggested that neither protein nor chitin accumulate copper cations to any great extent in *Ganoderma lucidum* cells. They deproteinated cell walls by alkali extraction, which marginally increased the binding of copper by the cell walls. However, as later experiments in the present study will show, treatment with hot alkali may in fact increase the total binding capacity of the cell wall components (i.e. both the alkali soluble and insoluble fractions accumulate greater quantities of Cu^{2+}

after alkali treatment, see figure 9.4, chapter 9), and thus assay of metal binding by just the deproteinated cell wall is not sufficient grounds to exclude the accumulation potential of the protein component.

6.6 CELL WALL COMPONENT EXTRACTION

6.6.1 INTRODUCTION

Investigations of the process of metal accumulation by isolated cell wall components is one of the most used and successful techniques in studies of cell wall-metal cation binding studies. The aim of this section of the present research was to provide complementary data to that produced by the partial enzyme degradation of the cell wall components reported in the previous section. A cell wall component identified as being intrinsically involved in cation binding (as seen by decrease in metal accumulation by the partially degraded cell walls in the previous section) could be assumed to accumulate copper when isolated.

6.6.2 MATERIALS AND METHODS

(a) Mannan Isolation

Two alternative methods of mannan isolation were used (refered to as A and B).

(1) Preparation method A ($Mannan_A$): 500 mg of yeast isolated cell walls (i.e. 5 $mg.cm^{-3}$) was fractionated using alkali (0.75 $mol.dm^{-3}$

NaOH, 100 cm^3) at 75°C for 6 hours under nitrogen (Catley, 1988). After centrifugation (3 000 g x 10 min) the pellet was washed with water by centrifugation and the supernatants from the alkali treatment and the washes were combined and the mannans, soluble in this fraction, were precipitated using Fehlings solution (which contains $CuSO_4$), a mannan complexing agent. Copper was dissociated from the mannans using ethanol containing 5% (v\v) concentrated HCl, and the mannans were isolated by centrifugation (3 000 g x 10 min). The mannan- containing pellet was washed with ethanol and subsequently dialysed twice for 4 hours at 4°C against 1 dm^3 ultra-pure water, and freeze-dried.

(2) Preparation method B ($Mannan_B$): Mannan was extracted according to the method of Northcote and Horne (1952). Cell walls (150 mg) were digested with 3.1 cm^3 of 3% (w\v) NaOH for 6 hours at 100°C under nitrogen. The digested material was centrifuged (3 000 g x 10 min) and the supernatant was decanted, and simultaneously resuspended and acidified to pH 6.0 in 3.5 cm^3 of 2 $mol.dm^{-3}$ acetic acid. The mannan fraction was precipitated from the supernatant with 4 volumes of ethanol. The supernatant was discarded and the precipitate was resuspended in ultra-pure water and re-precipitated with ethanol overnight. The white solid precipitate was subsequently washed with ethanol and ether, dialysed twice for 4 hours at 4°C against 1 dm^3 ultra-pure water, and freeze-dried.

(b) Glucan Isolation

Two alternative methods of glucan isolation were used (refered to as A and B).

(1) Preparation method A ($Glucan_A$): The alkali insoluble pellet from the $mannan_B$ extraction was again digested with 3.1 cm^3 of 3% (w\v) NaOH for 6 hours at 100°C under nitrogen. The material was precipitated by centrifugation (3 000 g x 10 min) and the pellet extracted with 2.0 cm^3 of 0.5 N acetic acid (75°C for 6 hours under nitrogen) and centrifuged (3 000 g x 10 min). The supernatant was discarded and the pellet was washed with 2.0 cm^3 volumes of ethanol and ether to remove the lipids (Northcote and Horne, 1952) and then dialysed twice for 4 hours at 4°C against 1 dm^3 ultra-pure water, and freeze-dried.

(2) Preparation method B ($Glucan_B$): This method of obtaining glucan was that of Northcote and Horne (1952). Cell wall material (100 mg) was extracted with 5 cm^3 of 0.5 N acetic acid (75°C for 6 hrs under nitrogen). The insoluble fraction was centrifuged and re-extracted with acetic acid. After washing the pellet with water and centrifuging (3 000 x g for 10 min) to remove glycogen, it was washed with 5 cm^3 volumes of ethanol and ether, and then dialysed twice for 4 hours at 4°C against 1 dm^3 ultra-pure water, and freeze-dried.

(c) Chitin and Chitosan Extraction

Chitin and chitosan was extracted from 100 mg of freeze-dried yeast cell walls following the method of Muzzarelli *et al* (1980). The cell walls were de-mineralized with HCl for 5 hrs, using 11.8 : 1 (w\w) ratio of 5% HCl to dry mass of yeast cell walls. After washing the cell walls to neutrality with ultra-pure water, the solution was centrifuged (3 000 x g for 10 min), and the pellet was subsequently deproteinated with 2% NaOH (65°C for 2 hrs under nitrogen) and once

again washed to neutrality. The solution was centrifuged again and the pellet was then dialysed and freeze-dried. The alkali-heat treatment could have increased the ratio of chitosan to chitin in the preparation by deacetylation of the chitin to yield chitosan.

(d) Copper Binding to Extracted Yeast Cell Wall Components:

Copper binding during dialysis

The dialysis apparatus used was similar to that of Marrack and Smith (1932). Two identical perspex units, each contain five separate 1.2 cm^3 chambers were clamped together with a single layer of dialysis membrane between the two halves to give five 2.4 cm^3 chambers, each bisected by a semi-permeable membrane (Spectrapor dialysis tubing, 6 - 8 kDalton cut-off). Two small perspex balls were placed in each chamber to agitate the solution during shaking.

To one half of the chamber was added 1 cm^3 of 4 $mmol.dm^{-3}$ $CuCl_2$, while to the other side was added 1 cm^3 of 5 $mmol.dm^{-3}$ PIPES/TMAH buffer, pH 6.1. The time required for a copper ion concentration to reach equilibrium across the dialysis membrane was determined by comparing the copper concentration on both sides of the membrane periodically. Equilibrium was reached after 4 hours. These figures were also used to determine the quantity of Cu^{2+} which bound to the walls of the chambers and the dialysis tubing.

Binding studies

Comparative metal binding studies of the isolated yeast cell wall were conducted in the dialysis apparatus at 22°C in a shaking water bath. One cm^3 of 4 $mmol.dm^{-3}$ $CuCl_2$ solution was pipetted into one side of the chamber, while 1 mg of yeast cell wall material suspended in 1 cm^3

of PIPES/TMAH buffer was pipetted into the opposite side. After 4 hours a 0.1 cm^3 sample was removed from the compartment of each chamber into which the $CuCl_2$ was initially added, diluted to 1 cm^3, and the amount of free Cu^{2+} determined using flame atomic absorption spectroscopy. The amount of Cu^{2+} bound by the cell walls could then be determined by subtraction of the free Cu^{2+} from the total Cu^{2+}.

6.6.3 RESULTS

The accumulation of copper by the extracted cell wall components can be seen in figure 6.15 and table 6.6.

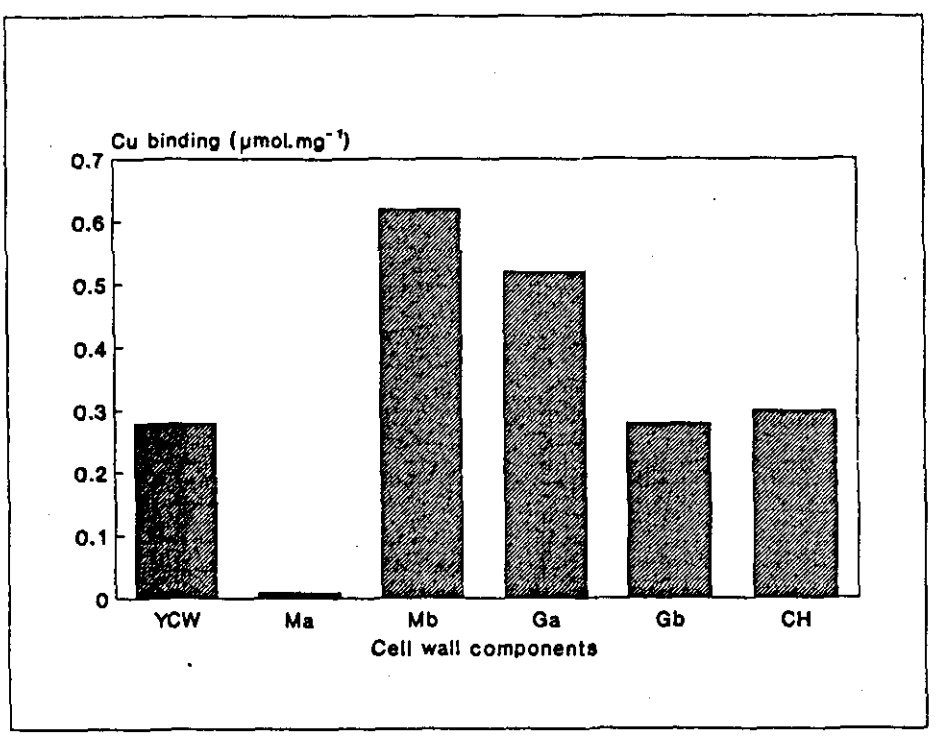


Figure 6.15: Copper uptake by extracted macromolecular components of the yeast cell wall. Unmodified yeast cell walls (YCW), Mannan preparations A and B (Ma and Mb), Glucan preparations A and B (Ga and Gb) and Chitin/chitosan preparation (CH).

TABLE 6.6: Parameters of copper binding by the cell wall of *S. cerevisiae*.

Sample	Protein/ carbohydrate ratio	Copper binding $\mu mol.mg^{-1}$ (Cu/mass extract)
Cell wall	0.159	0.28 ± 0.096
$Mannan_A$	0.036	0.00 -
$Mannan_B$	0.028	0.62 ± 0.105
$Glucan_A$	0.286	0.52 ± 0.130
$Glucan_B$	0.179	0.28 ± 0.130
Chitin	0.001	0.30 ± 0.020

In these results there does not appear to be a direct relationship between the protein content of the extract and its ability to accumulate copper. The greater quantity of protein in the $Glucan_A$ preparation compared to the $Glucan_B$ preparation is reflected in greater uptake of copper by $Glucan_A$. However, $Mannan_B$, which has a relatively high carbohydrate content, binds the most copper. Phosphate groups may still be associated with extracted mannan, and these could be partially responsible for the high level of metal binding. However, the carbohydrate moiety itself may be responsible for a considerable amount of the metal binding. It is clear that many of the cell wall components are capable of binding copper in relatively similar quantities. However, their binding affinity varies (figure 6.16; table 6.7). Chitin and chitosan are known to be capable of binding copper (Muzzarelli, 1977), so it is not surprising that this extract was capable of binding large quantities of metal in spite of a low protein content.

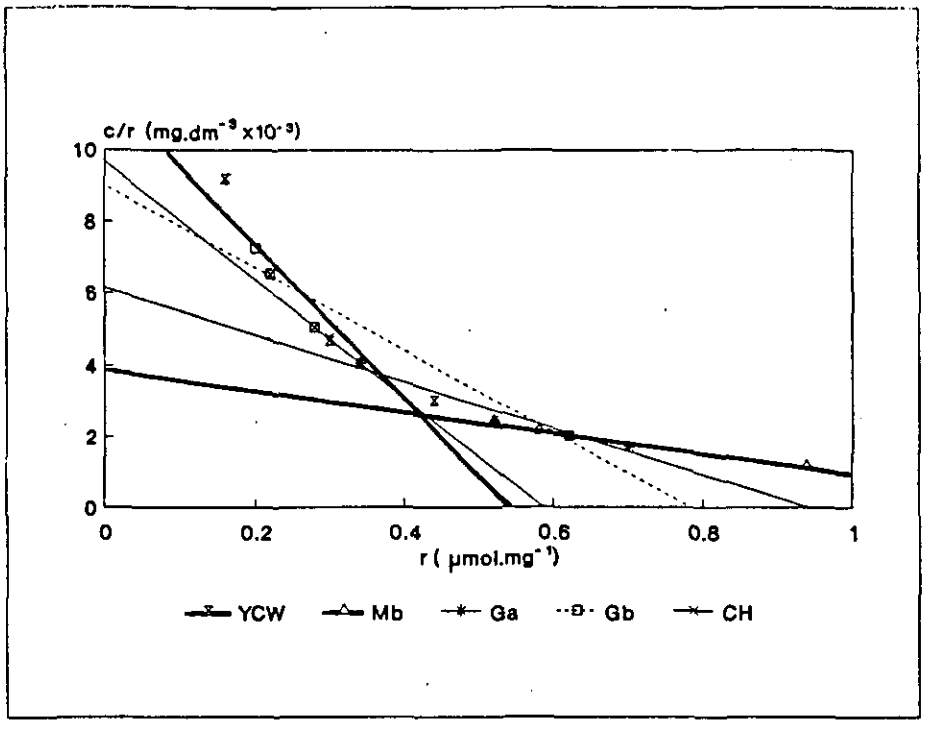


Figure 6.16: Inverted Scatchard plot of copper binding by cell wall extracted components. Unmodified yeast cell walls (YCW), Mannan preparation B (Mb), Glucan preparations A and B (Ga and Gb) and Chitin/chitosan preparation (CH).

TABLE 6.7: Affinity of the extracted cell wall components for copper.

Sample	Maximum Cu binding ($mmol.mg^{-1}$ yeast)	K_D ($mol.dm^{-3}$)	K_A ($dm^3.mol^{-1}$)
Cell wall	0.53	2.03	0.49
$Mannan_B$	1.36	0.28	3.57
$Glucan_A$	0.94	0.71	1.41
$Glucan_B$	0.77	1.27	0.83
Chitin	0.58	1.71	0.58

Maximum Cu binding (mol $Cu^{2+}.g^{-1}$ yeast $x10^4$) determined from the X-intercept.
Dissociation constants (K_D) were determined from the slope of the graph.
Association constants (K_A), are the inverse of the dissociation constants.

6.6.4 DISCUSSION:

Other studies found that after bioaccumulation of copper, cadmium and cobalt by *S. cerevisiae*, most metal was found to be bound to the cell wall (White and Gadd, 1986), and therefore metal adsorption to the cell can be considered as a surface-related phenomenon (Schenck *et al*, 1988).

Cell surface areas can be calculated from the measured cell diameters. In this study the scanning electron micrographs of whole yeast cells shown in chapter 5 were used for these calculations. The diameter of stationary phase yeast cells was measured as 5 μm. From this the cell surface area can be calculated:

$A = 4 \pi r^2 = 4 \times 3.14 \times 2.5 \times 10^{-6}$ m $= 7.9 \times 10^{-11}$ m^2

Also 2×10^8 cells.cm^{-3} = 15.8 mg dry mass

The surface area of one milligram dry mass of yeast cell walls can therefore be calculated as: 1 mg dry mass = 1.27×10^7 cells which is equivalent to: $1.27 \times 10^7 \times 7.9 \times 10^{-11}$ m^2 = 1×10^{-3} m^2.

The cell wall binds up to 0.5 μmol (0.5×10^{-6} mol) of copper per mg. Therefore the cell wall binds approximately 5×10^{-4} mol of Cu^{2+} per m^2.

In terms of the relative importance of the cell wall in bio-accumulation, they bind approximately 0.4 μmol (400 nmol) of copper per mg dry mass at the $CuCl_2$ concentrations assayed here (see table

6.3 in section 6.3.3). Whole cells bind 100 nmol Cu^{2+} per mg dry mass when the cell concentration in suspension is 1 mg dry mass cm^{-3} and the ambient copper chloride is 200 $\mu mol.dm^{-3}$ (calculated in chapter 2).

Since the cell wall is approximately 25% of the cell dry mass, this means that the cell wall must bind 400 nmol x 25%, *i.e.* 100 nmol of Cu^{2+}. In other words the cell wall binds almost all of the accumulated copper when the copper to cell biomass ratio is low. This in turn means that in the accumulation curves where there is an initial rapid accumulation step this probably signifies binding of metal to the cell wall.

Beveridge (1986) proposed that not all metal binding by the cell wall was the result of cation coordination by ligands, and that the initial stoichiometric binding was followed by another step where the original binding site becomes a nucleation site for deposition of further metal, until the deposit is physically constrained by the structure of the cell wall. This mechanism would lead to large deposits which would be difficult to remove. It was suggested that carboxyl groups, but not phospho-diester groups, served as nucleation sites on bacterial cell walls. This may also be true of yeast cell walls. If this is so then the number of Cu^{2+} binding sites calculated in section 2.4 must be revised.

6.7 BIOSORPTION OF METALS BY BACTERIAL CELL WALLS

The binding of cations by bacterial cell walls has been studied in greater detail than those of yeasts, and bacteria as a group also possess a wider range of different cell wall components. A comparison of the data on metal accumulation by bacteria with that on yeast could therefore be enlightening.

6.7.1 Gram Positive Bacterial Cell Walls:

Isolated cell walls from *B. subtilis* accumulate various cations, the order of capacity for binding being:

$$Fe^{3+} > Cu^{2+} > Na^{+} > Ca^{2+},\ Au^{2+} > Ni^{2+} > Hg^{2+},\ Ag^{+},\ Pb^{2+} > Co^{2+}$$

Of these, gold is unusual in that it may form crystals and therefore be accumulated in quantities in excess of cell wall binding sites. Iron has a unique and complex interaction with water and therefore may bind in a different manner to other cations (Beveridge and Murray, 1976).

There are two major polymers in the cell wall of Gram positive bacteria; these are teichoic acid and peptidoglycan. Peptidoglycan is a heteropolymer of repeating dimers. One of the monomers of the dimer is *N*-acetylglucosamine, which is the sole monomer found in chitin in yeasts. Partial digestion of the walls with lysozyme (which degrades peptidoglycan) decreases the affinity of the cell wall for some cations but not others, suggesting a degree of preferential binding for some polymers. An alternative suggestion is that lysozyme converts the relatively condensed cell wall into a more open

structure (Beveridge and Murray, 1976). In a number of Gram positive bacteria it was determined that magnesium or calcium cations can each pair with two ionic groups in the cell wall, although at high ionic strength each cation may only bind to one cell wall anionic group each (Marquis et al, 1976). The amount of magnesium bound by these bacteria was generally inversely related to the compactness of the structure of the cell wall (Marquis *et al*, 1976).

Teichoic acid, like the manno-protein fraction of the yeast cell wall, contains phospho-diester links. Extraction of teichoic acid caused a stoichiometric reduction in monovalent cation binding. Almost total removal of teichoic acid resulted in the elimination of calcium binding, but the binding of transition metal cations was only reduced by about 50%. Monovalent alkali metals apparently bind to teichoic acid in equimolar quantities, while the divalent ions, due to their higher valency, required two phospho-diester residues per ion for binding (Beveridge and Murray, 1980).

B. subtilis cell walls possess a number of chemical groups which may be involved in cation binding. Amino nitrogens and hydroxyl groups are available for complex formation, while carboxyl groups and phosphates are sites for ion exchange. There is experimental evidence in favour of ion exchange being the main process involved in metal cation binding (Weppen *et al*, 1990), although data from the fungus *Rhizopus arrhizus* suggests that complex formation is dominant during binding (Tobin *et al*, 1990). It is possibly the spacial arrangement of the coordinating groups, such as amino groups, that is responsible for the strength of metal cation-cell wall bindings site to a large degree (Doyle *et al*, 1980). Binding is only partially related to a number of

factors such as metal ion charge, ionic radius, atomic number, and heat of hydration (Beveridge and Murray, 1976).

Another Gram positive bacterium, *B. licheniformis* has a different cell wall composition from that of *B. subtilis*. Its cell wall contains relatively less peptidoglycan and more teichoic acid than that of *B. subtilis*, and also contains some teichuronic acid. Most of the cation binding in this species has been found to be by the teichoic and teichuronic acids (Beveridge, 1986), and there is an overall lower quantity of the metal binding compared to *B. subtilis* cell walls. *Sporosarcia ureae* contains no teichoic acid in the cell wall but does have a peptidoglycan layer and an outer protein layer. This protein layer has been shown to be capable of accumulating 6.5 $\mu mol.mg^{-1}$ dry mass of magnesium, which is a relatively high level of accumulation (Beveridge, 1986).

Comparison of the importance of proteins for metal binding in yeast and *B. subtilis* is not possible as there is little or no protein in the *B. subtilis* cell wall (Doyle *et al*, 1980). However, peptides are found in the peptidoglycan component, but these simple peptides would not provide the range of chemical binding groups and three dimensional arrays of ligands that proteins could.

6.7.2 Gram Negative Bacterial Cell Walls:

Purified envelopes of Gram negative bacteria are capable of binding a range of metal cations (Beveridge and Koval, 1981). Isolated Gram negative envelopes, however, appear to bind only one tenth of the metal which Gram positive cell walls are capable of binding, possibly owing to the higher charge capacity of the latter. Gram negative

bacterial cell walls have a much thinner layer of peptidoglycan than do Gram positive bacteria, there is no teichoic acid present, and they possess a distinctive outer lipid membrane. Binding occurs mainly to the peptidoglycan layer although there is limited binding to the phosphate groups of the phospholipids of the outer membrane (Beveridge, 1986). There is only very limited binding of alkali and alkaline-earth metals by Gram negative bacterial cell walls (Beveridge and Koval, 1981).

It appears thus that the peptidoglycan component of bacterial cell walls binds cations, as does teichoic acid although it has fewer binding sites. It is also possible that protein may bind more than either of these components, but the composition of active groups in the protein and their three dimensional arrangement (and thus their coordination properties) varies with the amino acid composition of the protein, which in turn means that metal binding to a particular protein type will depend on the protein's structure. If similar patterns of cation binding occur with yeast cell walls as they do in their bacterial counterparts then this would suggests that chitin, chitosan, protein and the phosphate groups of phosphomannans are the most likely candidates for cation binding in the yeast cell wall.

6.8 CONCLUSIONS

The experimental results presented in this chapter should provide a comprehensive understanding of the nature of metal cation binding by the yeast cell wall.

The results of chemical modification of the cell wall suggest that three of the most important chemical ligands for copper were carboxyl, amino, and hydroxyl groups. Hydroxyl groups, which are found on the carbohydrate polymers, far outnumber the other two groups and yet exert at best a similar level of binding, suggesting that they are individually poor binders of metals, but owing to their predominance, hydroxyl groups must be considered as important in metal binding. The carboxyl groups are found on the side chains of acidic amino acids, while the amino groups are found as part of both proteins and chitosan.

Enzymatic degradation of the yeast cell wall provided an indication that there is only a limited relationship between protein content and metal binding capacity of the cell wall. This suggests that protein, although an important metal binding component, can only be considered to be marginally more important than the other components. There was, however, a distinct relationship between the extent of degradation of the cell wall and the metal binding capacity of the residue. The literature suggests also that protease degradation, which caused the greatest loss of metal binding capacity, is capable of removing the outer layer of the cell wall to leave only glucans and some chitin and chitosan (Hunter and Asenjo, 1988).

Analysis of copper binding by polymers extracted from the yeast cell wall indicates that mannans are the most significant accumulators of protein, both in quantity and affinity. Phosphate quantities in the extract were not accurately measured owing to insensitivity of the assay used (and therefore it is not described); but if the phospho-diester bridges remain between the mannans, then these may be

responsible in part for the high metal binding capacity of this fraction.

Comparison with the literature available on metal binding by bacterial cell walls suggests that the proteins and phosphate groups in the phosphomannans should be the most important metal chelators in the yeast cell wall.

Any extracellular metal cation that passes through the cell wall without being bound comes into contact with the cell membrane. This membrane controls to a large extent the intracellular concentrations of various cations. The next two chapters deal with this membrane control and the metal binding molecules compartmentalized within the cellular membranes.

7. INTERNALIZATION OF HEAVY METALS BY YEAST

7.1 INTRODUCTION

A unique attribute of living cells is their ability to maintain an electrochemical disequilibrium between the intra- and extracellular environment. In fact a substantial amount of the cell's energy supply is spent on maintaining this disequilibrium. This disequilibrium is possible because cell membranes (phospholipid bilayers) are electrical insulators and physical barriers to the environment. They compartmentalize the cell from the extracellular environment and create micro-environments within the cell.

The lipid membranes are selectively permeable towards specific ions. They are most permeable to small inorganic monovalent ions, considerably less permeable to multivalent ions, and generally totally impermeable to complex organic ions, such as polyphosphates, and to proteins. However, these latter two groups constitute a substantial part of the intracellular ionic strength (Benedek and Villars, 1979).

The quantity of free ions found within a cell is dictated by the ratios of charged groups across the membrane. The ratio of diffusible cations on one side of the membrane to the other is inversely proportional to the ratio of the anions on the two sides (1 and 2), *viz*:

$$\frac{[X^+]_1}{[X^+]_2} = \frac{[Y^-]_2}{[Y^-]_1} = r$$

where $[X^+]$ and $[Y^-]$ are the concentration of free cations and anions, respectively. This ratio (r) is referred to as the Donnan constant. The energy required (ΔG) to transport cations across the membrane and into the cell (compartment 2) is:

$$\Delta G = RT \times \ln \frac{[X^+]_1}{[X^+]_2} + ZF\Delta\Psi$$

Where R is the universal gas constant, T is the absolute temperature, F is Faraday's constant (9.65 $kJvolt^{-1}.mol^{-1}$), Z is the charge on the ion and $\Delta\Psi$ is the membrane potential in volts. Since the ions in this equation are free, intracellular chelation of ions reduces the energy requirement for cation transportation.

Intracellular anions that cannot leave the cell owing to large size or other factors constitute an intracellular non-permeant anion pool. The intracellular non-permeant anion equivalent concentration has a typical value of 125 $meq.dm^{-3}$. As these are intracellular anions they comprise a part of the component $[Y^-]_2$.

The generally low permeability of lipid membranes to metal ions means, however,that the uptake of metal ions normally occurs *via* facilitated transport (Schenck *et al*, 1988). Within the membranes there exist transport channels, usually of a protein or glycoprotein nature, which control the influx and the efflux of molecules across the membranes. Some uptake systems are very specific. For instance, the nickel uptake system in the bacteria *Methanobacterium bryantii* is unaffected by high concentrations of a range of monovalent and divalent ions except for Co^{2+} (Jarrell and Sprott, 1982).

The transport system involved in metal ion accumulation is similar to active transport systems in that it shows saturability and specificity Fuhrmann and Rothstein, 1968). It is metabolically-dependent and assymetric (high influx with low efflux), and permits competition of pairs of co-ions. Metal accumulation is, however, not affected by whether the cell is utilizing aerobic or anaerobic metabolism (Fuhrmann and Rothstein, 1968). This fact raises the possibility that if the very different energy outputs of these two metabolic pathways is not important, then perhaps cation uptake is not directly linked to intracellular energy economy.

In their ionic form heavy metals may be contained by the cell membrane or by intracellular membrane organelles. An extreme case of this can be seen in certain bacteria which are capable of synthesizing magnets of magnitite particles [Fe_3O_4] within intracellular membranous organelles. These magnets are believed to be used by the microbes to navigate to the sediment of natural freshwater bodies (Matsunaga, 1991).

7.1.1 The Plasma Membrane:

The composition of the lipid membranes of *S. cerevisiae* have been studied in detail (Rose and Veazey, 1988) and appears to be very similar to the membranes of other cells. The role of the membranes in metal binding and accumulation is, however, still relatively obscure. It is believed that zinc cations are involved directly in the maintenance of both membrane structure and function. Zinc is apparently mainly associated with the proteins found in the lipid bilayer and zinc is at least partly responsible for the permeability of the membrane to potassium ions (Bettger and O'Dell, 1981). A low

extracellular zinc concentration results in loss of plasma membrane-associated zinc, which consequently results in destabilization of the membrane. Zinc has a unique role to play in membrane integrity which cannot be performed by other ions (Bettger and O'Dell, 1981).

Yeast cell plasma membranes may be artificially permeabilized by chemicals such as cetyltrimethylammonium bromide (Naina *et al*, 1991) or polymers such as chitosan (Joho *et al*, 1985), but they may also be permeabilized by certain cations. The addition of copper ions to cell suspensions of yeast may lead to increased permeability of the plasma membrane with massive and rapid release of 70% of the cellular potassium (Oshumi *et al*, 1988). The Cu^{2+} causes selective lesions of the permeability barrier of the plasma membrane but does not affect the permeability of the vacuolar membrane (Oshumi *et al*, 1988). This was evident from the fact that glutamic acid, an amino acid which is found mainly in the cytoplasm, was rapidly released from the cell (within the first four minutes of contact), while arginine, an amino acid that is stored in the vacuole, remained within the cell. Equal concentrations of zinc cations did not result in similar loss of potassium or glutamic acid, and therefore zinc probably does not permeate the membrane.

Heavy metal cations may be taken into membrane-bound non-exchangeable pools by yeast cells. *S. cerevisiae* cells adapted to cultures containing copper, cadmium and cobalt salts internally accumulated metals (White and Gadd, 1986). The uptake of some metals is enhanced by addition of glucose, especially in the presence of inorganic phosphate (Jones and Gadd, 1990). Uptake is diminished by the presence of H^+ at low ambient pH, but a direct cation/H^+ exchange system does not appear to

be involved (Jones and Gadd, 1990). In some cases two potassium ions (or two sodium ions in the case of Na^+ loaded cells) are released for every divalent cation absorbed (Fuhrmann and Rothstein, 1968), but as will be seen later this is not a general rule.

Certain metal ions such as iron (Raguzzi *et al*, 1988; Lesuisse *et al*, 1987, 1990; Lesuisse and Labbe, 1989) and copper are reduced by a transplasma membrane redox system to divalent and monovalent ions respectively before uptake into yeasts with intact membranes. Hg^{2+} associates with cell membranes to a greater extent than other heavy metals. The yeast *Cryptococcus albidus*, when cultured in the presence of mercuric chloride, was found to accumulate mercury in both the cell wall and membranes. Mercury bound to the plasma membrane and other cellular membranes such as the inner and outer mitochondrial membranes, the nuclear membranes, the endoplasmic reticulum and various other vesicles (Brown and Smith, 1977).

Nickel uptake by *Methanobacterium bryantii* could be increased by artificially imposing a pH gradient (inside alkaline) upon it by adding acid to the cell suspension. The outcome of the experiment implies that nickel transport is not necessarily dependent on the membrane potential or intracellular ATP levels, but is coupled to proton gradients and possibly proton movements (Jarrell and Sprott, 1982). It should be noted however that the stimulated uptake was transitory and the majority of the nickel was soon lost to the extracellular solution.

Non-voltage gated K^+ selective channels are present in the yeast plasma membrane (Van der Mortel *et al*, 1990). At neutral pH, addition

of glucose to an anaerobic suspension of non-metabolizing yeast cells results in a transient net efflux of K^+ from the cells via these gates, and a concomitant transient hyperpolarization of the plasma membrane. These responses are both suppressed by low concentrations of multivalent cations which block these ion gates. This hyperpolarization only occurs above pH 5.5 (Van der Mortel *et al*, 1990).

Glucose addition may also cause transient influxes of calcium cations into *S. cerevisiae* cells. The glucose-mediated calcium influx is at least partially independent of cellular cAMP levels, while intracellular acidification may be responsible for part of the glucose stimulation of Ca^{2+} influx (Eilam et *al*, 1990). The accumulation of calcium by the cell is a two step process. Initially there is an influx of Ca^{2+} across the plasma membrane; this step proceeds down an electrochemical gradient for Ca^{2+} and is driven electrophoretically by the membrane potential. This initial step is completed within 20 seconds. The second step involves accumulation of Ca^{2+} into the vacuoles *via* an nH^+/Ca^{2+} antiport which itself depends on the vacuolar H^+ATPase activity, and is therefore energy dependent. This latter step is slower that the initial step and occurs within 3 minutes (Eilam and Chernichovsky, 1987).

The fate of calcium (and possibly other metal cations) after entering the cytosol is therefore eventually to be taken up into an intracellular membrane bound organelle, the vacuole.

7.1.2 The Vacuole:

Fungal vacuoles are multifunctional, intracellular membrane-bound organelles. They are internally acidic and contain a range of hydrolytic enzymes for degrading macromolecules. They also function as the main storage site for a number of metabolites and cytosolic ions as well as being involved in cytoplasmic pH homeostasis (Klionsky *et al*, 1990).

The cell is obliged to regulate its cytosolic ion concentration for several reasons. Some ions, such as Co^{2+} and Pb^{2+}, are potentially harmful and cannot be allowed to accumulate within the cytosol and must be transferred elsewhere. Other ions which are physiologically useful, such as Ca^{2+}, Mg^{2+}, and Zn^{2+}, may become toxic in excess quantities and therefore the cytosolic concentrations must be controlled in this case too (Klionsky *et al*, 1990). Also, some ions are involved in metabolic regulatory processes within the cell, demanding exact control of the concentrations of these ions (Hoober and Phinney, 1988). Storage of these ions in the vacuole allows for their immediate mobilization into the cytosol when required and is a source of these ions when their availability in the external milieu is temporarily limited.

Like the plasma membrane the uptake of certain ions by the vacuole is selective. For instance potassium and sodium ions are found in much greater quantities in the cytosol than they are in the vacuole (Klionsky *et al*, 1990) although K^+ may be accumulated in the vacuole at certain stages in the yeast life cycle (Okorokov *et al*, 1980). The vacuole appears to be the major site of intracellular iron storage in

the yeast cell (Raguzzi *et al*, 1988). Vacuoles are also the main compartments of intracellular magnesium (Okarokov *et al*, 1980) while calcium ions are also concentrated in the fungal vacuole (Cornelius and Nakashima, 1987).

Studies of zinc distribution in *S. cerevisiae* cells after uptake showed that within the cell, 56% of the zinc was to be found in the soluble vacuolar fraction, 39% was bound to insoluble components, and only 5% was free in the cytosol. The vacuoles, when isolated, were found to possess an ATP-dependent Zn^{2+} uptake system which may represent a Zn^{2+}/H^{+} antiport (White and Gadd, 1987). These vacuoles occupy 25 - 95% of the fungal cell volume and are the largest organelles in yeast cells. Their primary role is to ensure homeostasis of ions and nutrients in the cytoplasm and to function as lytic or metabolic compartments for intracelluar digestion (Jones and Gadd, 1990).

Certain heavy metals may be taken up into the vacuole. S. cerevisiae cells adapted to cultures containing copper, cadmium and cobalt salts internally accumulated heavy metals. Cadmium and copper were internalized into the soluble fraction but were not specifically located in the vacuole. Cobalt accumulation in both tolerant and native cells occurred primarily in the vacuole (White and Gadd, 1986). Certain mutants of *S. cerevisiae* with defective vacuoles have been shown to be more sensitive to heavy metal poisoning by ions such as Hg^{2+}, Cd^{2+}, Fe^{3+} and Cu^{2+} when compared to the parental strain, indicating that vacuoles must be involved in neutralizing the toxic effects of these metal cations in normal cells. Even Ca^{2+}, which is usually stored in the vacuole, may be inhibitory to the growth of strains with

defective vacuoles (Kitamoto *et al*, 1988).

The vacuole is therefore intimately involved with the accumulation of many heavy metals within the cell. One of properties that allows vacuoles to accumulate metals is the presence of polyphosphate bodies within the vacuole.

7.1.3 Vacuolar Polyphosphate Bodies:

Polyphosphates are the only macromolecular anions discovered in the fungal vacuole to date and are unique to the vacuole (Vaughn and Davis, 1981; Urech *et al*, 1978). Under certain growth conditions polyphosphates can amount to 10.4% of the cell dry mass (Urech *et al*, 1978). Under the microscope they appear as globules, often referred to as volutin, which may be synthesized *in vitro* by simply mixing polyphosphate and cations such as Ca^{2+} (Miller, 1984).

Polyphosphates serve dual functions in the vacuole. Because they are polyanions they can coordinate with basic amino acids and inorganic cations and therefore act as storage macromolecules for these cations. Secondly, because they reduce the concentration of free inorganic phosphate, amino acids and inorganic cations, they greatly reduce the osmotic pressure within the cell (Klionsky *et al*, 1990). The cytosol and the vacuole are maintained isotonic by this means. The polyphosphates may be from 3 - 260 phosphate units long, although the exact sizes are difficult to determine owing to cellular phosphatase activity during polyphosphate isolation (Klionsky *et al*, 1990), and the variability in length of the polyphosphate chain depending on the yeast's stage in the growth cycle (Greenfield *et al*, 1987).

INTERNALIZATION OF HEAVY METALS

Polyphosphates chelate metal ions through their negatively charged oxygen atoms. The chelating affinity of a polyphosphate chain increases with increasing chain length (Shumate *et al*, 1978). In *S. cerevisiae* 20 - 38% of the Ca^{2+} is trapped by polyphosphates (Ohsumi and Anraku, 1983). Cations other than calcium will form precipitates with polyphosphate (Miller, 1984) indicating similar chelation activity.

The polyphosphates therefore contribute to the anionic non-permeant concentration within the cell and, by chelating free cations in the vacuole, contribute to the maintenance of a concentration gradient of free cations into the vacuole.

In summary, the plasmalemma is capable of selectively accumulating metals by means of ion ports and effluxing certain unwanted ions. The membrane, then, acts as a barrier to the movement of ions and thereby ensures the maintenance of the disequilibrium. Metals entering the cell interior may bind to the membranes, remain free in the cytosol or be chelated to metallothioneins or other intracellular chelation agents. Alternatively,the metals may be taken up into intracellular organelles, particularly the vacuole where they may remain free or be complexed by polyphosphate bodies.

Certain cations, such as copper, may permeabilize the membranes, but this permeation is selective for membrane type. As has been stated previously, certain cations are selectively compartmentalized into the vacuole. By monitoring the release of these ions it is possible to determine which membranes are allowing transfer of ions, either by natural metabolic mechanisms or toxic effects.

7.1.4 Research Aims:

The aim of the present study was to determine whether intracellular cations are released from the cell during heavy metal accumulation. This in turn may yield further clues to the role of the plasma membrane and the vacuole in heavy metal bioaccumulation.

7.2 MATERIALS AND METHODS

The materials and methods used here are essentially those described in chapter 2. In this chapter, however, the loss of ions during the accumulation of copper was investigated. Potassium, magnesium, and calcium release into the medium was monitored during Cu^{2+} bioaccumulation by *S. cerevisiae* in various extracellular environments, such as differences in temperature, pH, and ionic concentration (the details of which are described in chapter 2). Metal cation accumulation was studied under non-steady-state conditions by the addition of external heavy metal salts (200 $\mu mol.dm^{-3}$ $CuCl_2$ except where stated) to native cells. Ca^{2+}, Cu^{2+} and Mg^{2+} quantities were determined by flame atomic absorption spectroscopy. Potassium levels were determined by flame emission spectroscopy. The absorbance of ultra-violet light (260 nm) by the extracellular solution was also followed during these experiments to monitor loss of organic compounds from the cell during metal accumulation.

The initial total cellular content of Ca^{2+}, Mg^{2+}, and K^{+} were determined, as were both the intra- and extracellular concentrations

of these ions after metal accumulation, to permit estimation of the percentage of the total of each intracellular cation released during the Cu^{2+} bioaccumulation process.

The release of intracellular cations from yeast when yeast cell suspensions were exposed to high levels of Cu^{2+}, Co^{2+}, or Cd^{2+} (500 - 2 500 μmol.dm^{-3} metal in 5 mmol.dm^{-3} PIPES buffer, pH 6.5) for 1 hour, and assayed for comparison. The possible inhibition of Cu^{2+} bioaccumulation by the presence of Ca and Mg was investigated by adding one of these alkaline-earth metals to the cell suspension 10 min. before addition of an equimolar concentration of Cu^{2+}.

7.3 RESULTS

During these experiments it became obvious that the pattern of release of intracellular cations during copper bioaccumulation differed between ions. It can be seen in figures 7.1 - 7.4 that potassium ions were rapidly lost from the cell during Cu^{2+} bioaccumulation while the loss of magnesium ions was far slower and more constant. Approximately 70% of the total intracellular potassium was released from the cell. There was a release of intracellular cations from the cells even in the absence of heavy metal cations, possibly as the result of a small percentage of yeast cells lysing in the low ionic strength solution.

Plots of intracellular cation release by yeast cell suspensions which do not include Cu^{2+} (referred to as native yeasts in figures 7.2 - 7.4) were included for comparison.

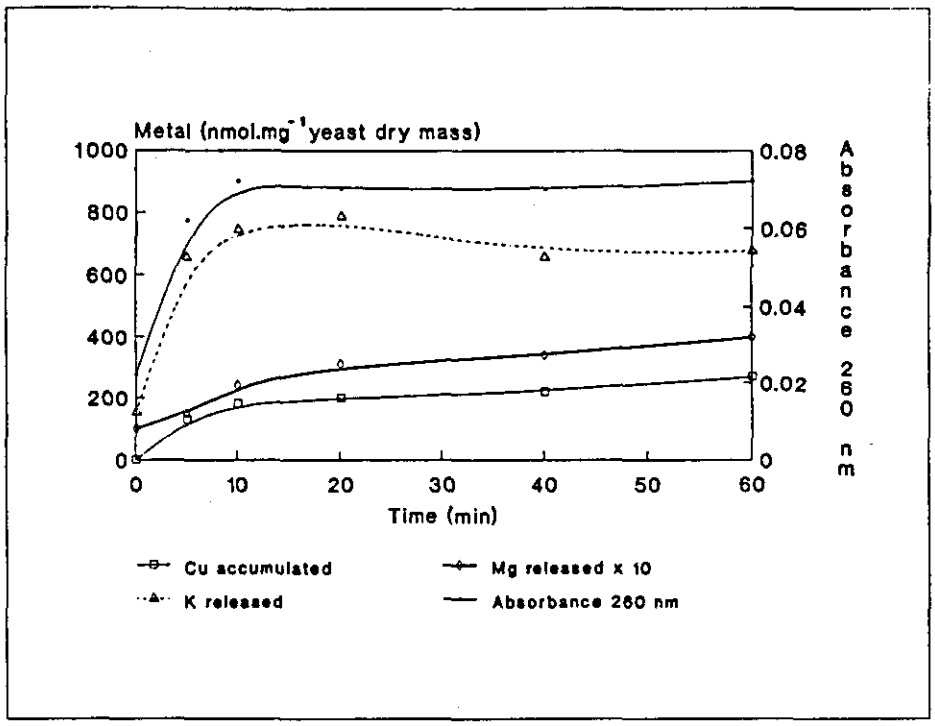


Figure 7.1: Release of intracellular cations and material that absorbs light at 260 nm during copper bioaccumulation.

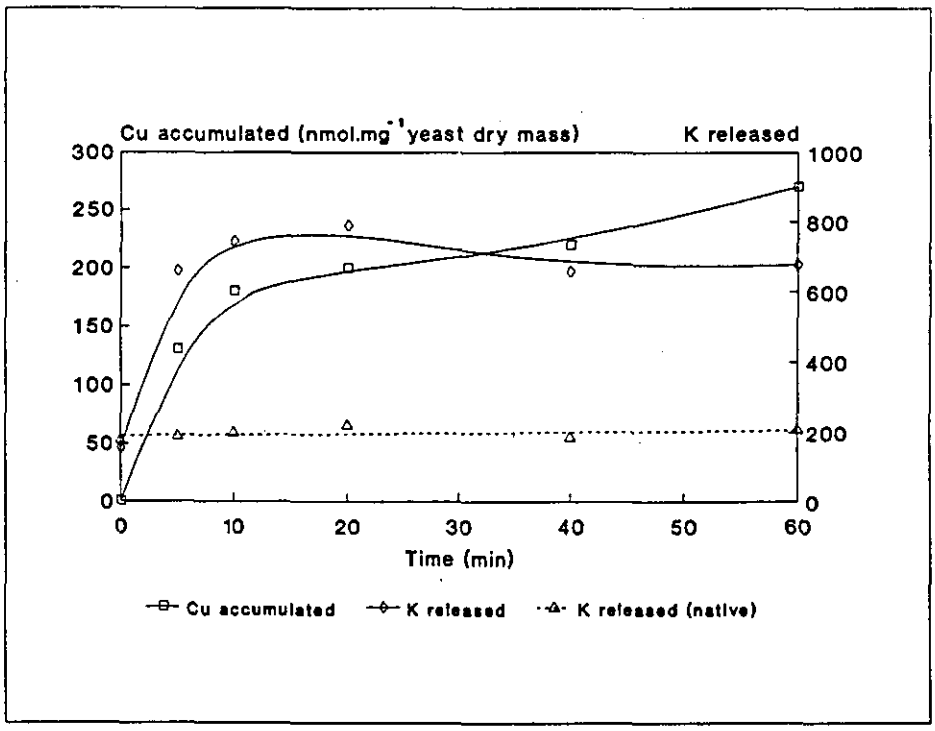


Figure 7.2: Release of intracellular potassium cations during copper bioaccumulation. Potassium in nmol.mg^{-1} cell dry mass.

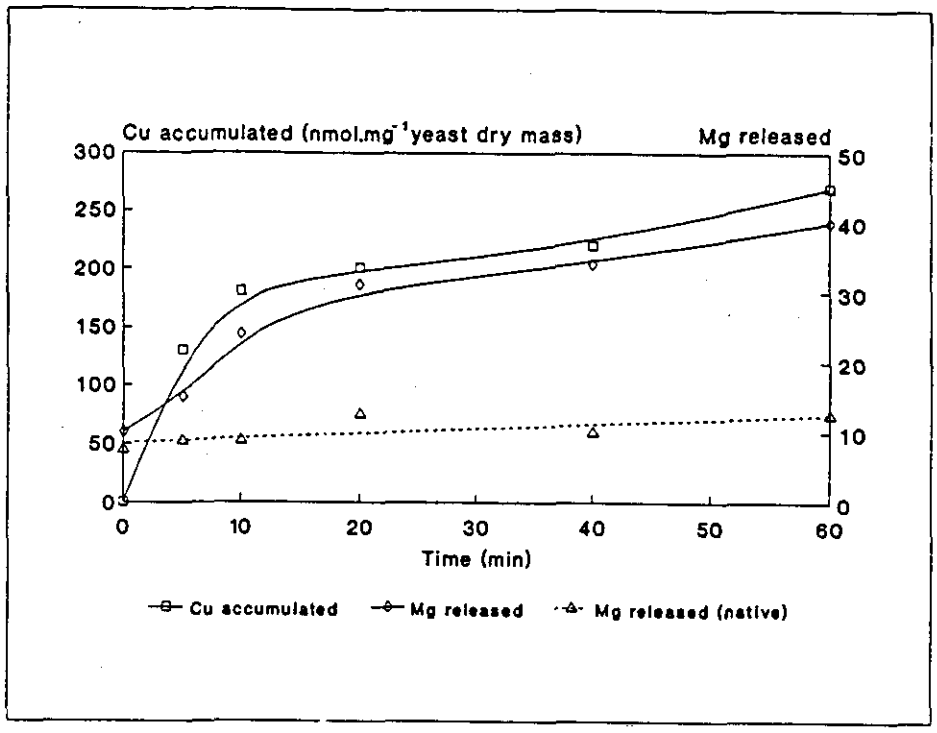


Figure 7.3: Release of intracellular magnesium cations during copper bioaccumulation. Magnesium in nmol.mg^{-1} cell dry mass.

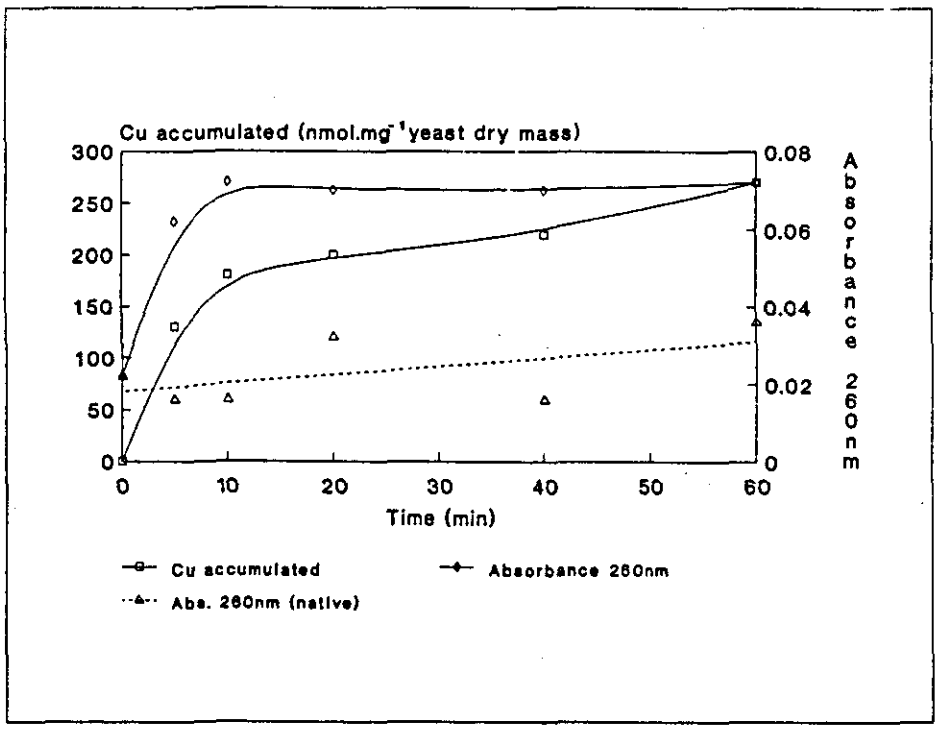


Figure 7.4: Increase of absorbance of the extracellular medium at 260 nm during copper bioaccumulation.

With increasing levels of copper accumulation (as a function of increasing external copper cation concentrations), there is a related loss of magnesium from the cell, although this loss is slightly more rapid at lower heavy metal concentrations (figure 7.5). The potassium loss is very different in that the maximum potassium loss occurs even at the lowest external copper concentrations used and does not increase with greater quantities of external copper cations (figure 7.6). Extracellular calcium levels varied little during these experiments (data not shown).

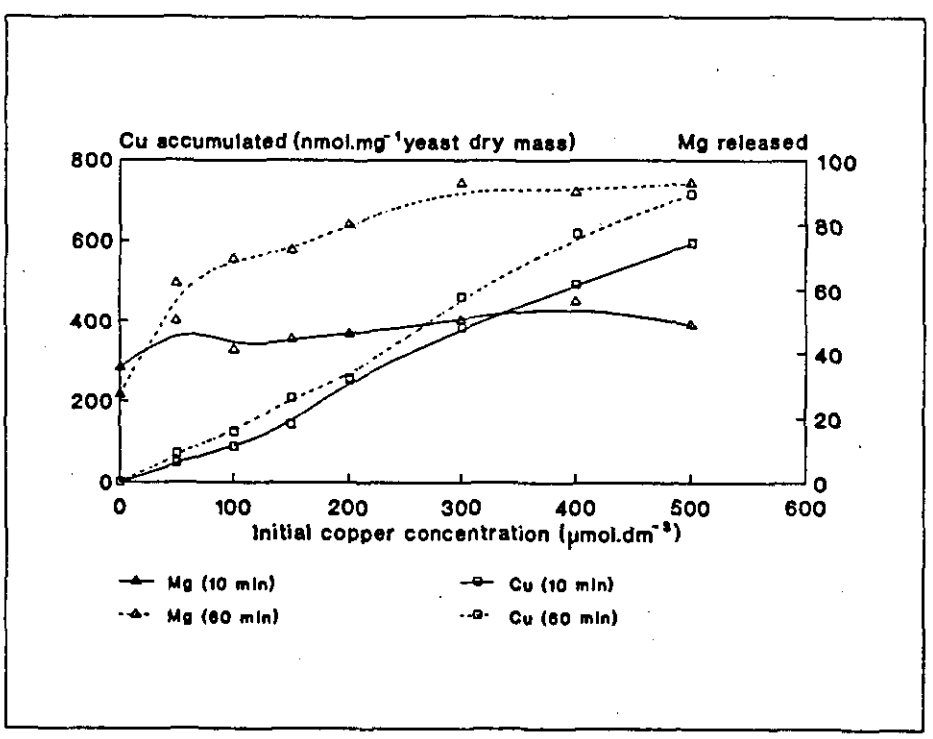


Figure 7.5: Release of magnesium ions during copper bioaccumulation after 10 and 60 min. of incubation as a function of initial copper concentration. Magnesium in nmol.mg^{-1} cell dry mass.

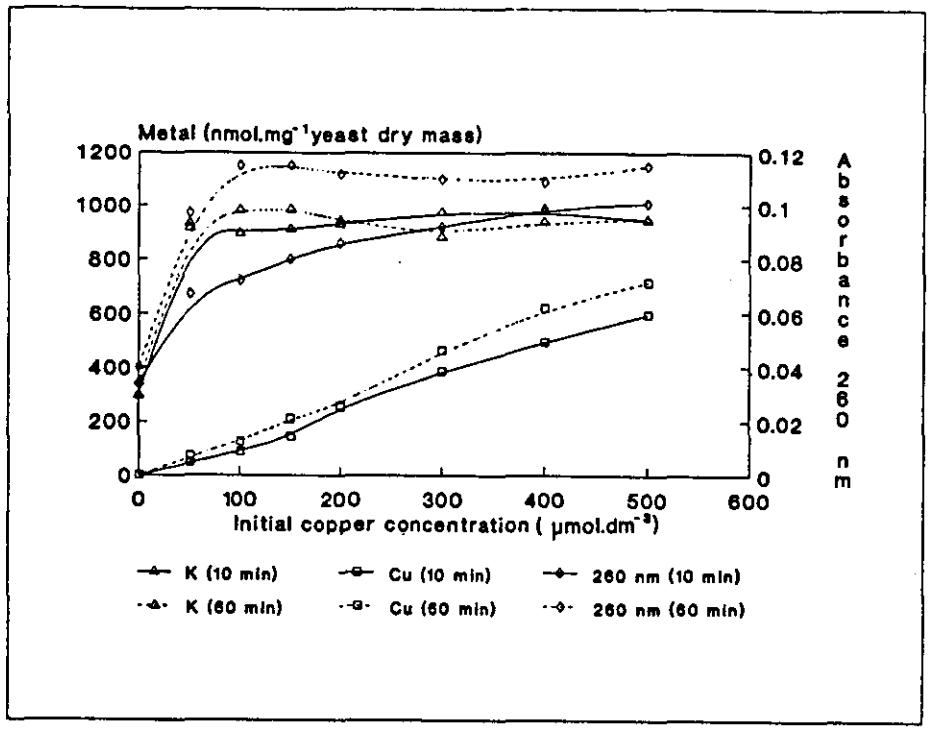


Figure 7.6: Release of potassium ions during copper bioaccumulation as a function of initial copper concentration at 10 and 60 min. of incubation time. Release of organic material (detected by absorbance at 260 nm) is included for comparison.

In figure 7.7 the relationship between magnesium release to copper accumulation at varying pH is clear. As copper accumulation increases there is a parallel initial release of magnesium ions to the extracellular solution. Also, at very high hydrogen ion concentrations, magnesium, along with calcium, is lost to the exterior. Calcium release is affected by pH to a far greater extent than any other variation of extracellular environment used in this study.

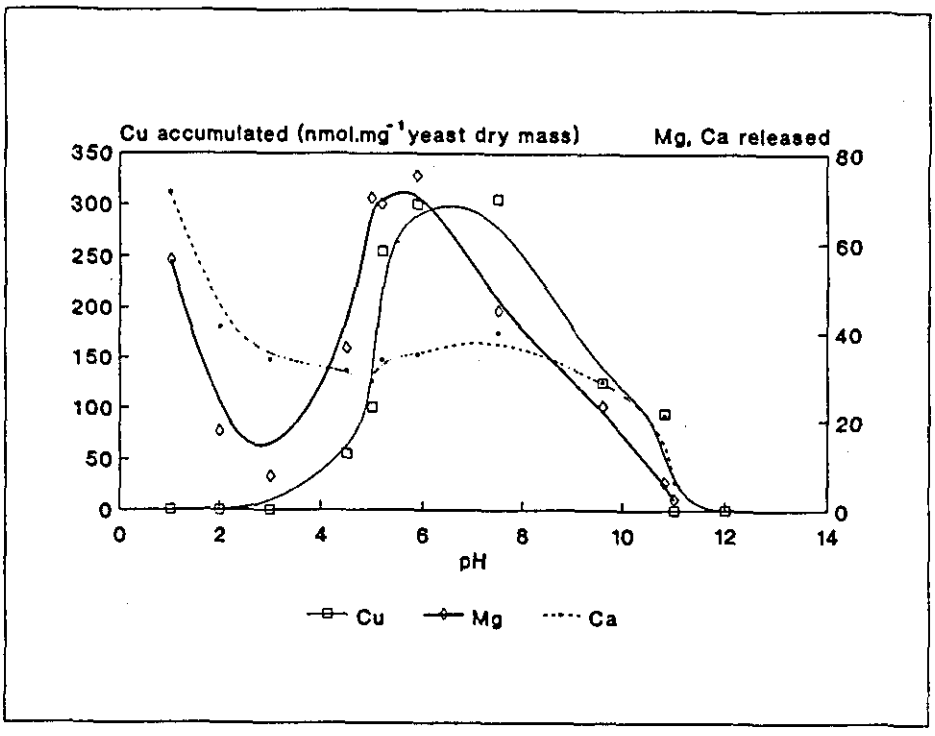


Figure 7.7: Magnesium and calcium release during copper bioaccumulation at various pHs. Magnesium and calcium in nmol.mg^{-1} cell dry mass.

The release of potassium during copper bioaccumulation was relatively constant over a range of temperatures, i.e. 5 - 40°C (data not shown). Magnesium losses increased with temperature rises and paralleled copper accumulation (figure 7.8), but magnesium release increased even when copper accumulation started to decline slightly at 40°C. This may reflect the greater fluidity of the lipid membranes at higher temperatures and therefore an inability to retain ions.

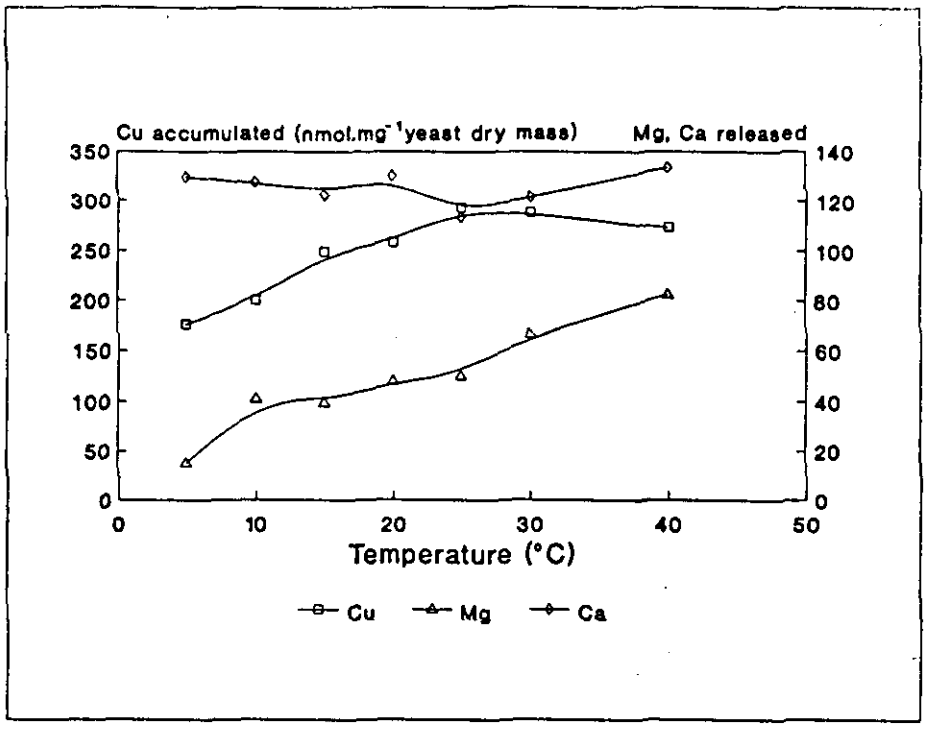


Figure 7.8: Intracellular cation release during copper bioaccumulation at various temperatures.

High external ionic strength (in the form of sodium chloride) decreased Cu^{2+} accumulation (by about 20%) but also caused stimulation of Cu^{2+}-induced release of intracellular cations. Magnesium was most affected, calcium release was the next greatest, while potassium release was affected least of all (66%, 52%, and 20% increases in release, respectively. The release of magnesium and calcium during Cu^{2+} accumulation is presented in figure 7.9.

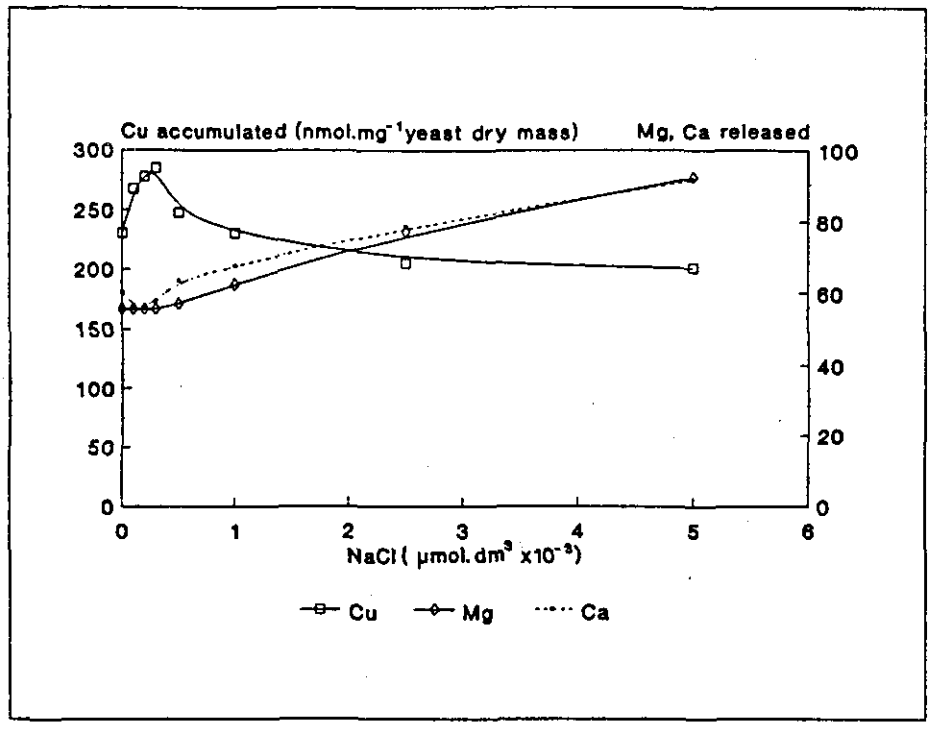


Figure 7.9: The release of cations during copper bioaccumulation from saline solutions.

In table 7.1 are presented the results of two separate experiments on bioaccumulation of heavy metals by *S. cerevisiae* cell suspensions. Unlike the other results presented in this section the residual intracellular metal concentration is reported rather than the concentration of metal released into solution. From the results presented in table 7.1 it is evident that there is a relationship between heavy metal accumulation and loss of intracellular potassium and magnesium, but not with calcium, at these heavy metal concentrations. Although heavy metal accumulation appears to increase extracellular release of material that absorbs at 260 nm, at higher concentrations of cadmium and cobalt this trend appears to be reversed. Intracellular concentrations of calcium and magnesium may increase in the presence of heavy metals when magnesium or calcium are

added to the cell suspension (500 $\mu mol.dm^{-3}$). In contrast much greater concentrations of calcium (2 500 $\mu mol.dm^{-3}$) in the cell suspension are required for accumulation of calcium in the absence of heavy metals. This suggests that the heavy metals affect the permeability of the yeast cell's plasma membrane to cations. Cu^{2+} is readily accumulated, Cd^{2+} less so, and Co^{2+} to an even lesser extent at these concentrations; these results are comparable to those of accumulation of these three metals by immobilized *S. cerevisiae* as reported in chapter 4).

TABLE 7.1: Yeast intracellular cation concentrations after heavy metal accumulation.

Metal added ($\mu mol.dm^{-3}$)	Residual intracellular metal conc. ($nmol.mg^{-1}$)			Extra-cellular absorbance at 260 nm	Heavy metal accumulation ($nmol.mg^{-1}$)
Experiment 1	Ca^{2+}	Mg^{2+}	K^{+}		
Control	65	75	370	0.024	0
Cu 500	65	43	43	0.063	675
Cu 500 + Mg 500	63	88	78	0.088	675
Cu 500 + Ca 500	88	35	43	0.083	725
Cu 1000	60	38	83	0.105	1 335
Cd 500	65	40	80	0.074	150
Co 500	80	78	380	0.025	20
Ca 500	60	75	375	0.027	0
Experiment 2	Ca^{2+}	Mg^{2+}	K^{+}		
Control	80	87	383	0.043	0
Cd 500	85	28	93	0.105	181
Cd 2 500	83	28	78	0.025	350
Co 500	93	90	370	0.065	25
Co 2 500	110	53	245	0.025	138
Ca 500	93	88	395	0.031	0
Ca 2 500	120	95	468	0.031	0

7.4 DISCUSSION

7.4.1 Intracellular Cation Release During Heavy Metal Bioaccumulation:

Potassium and sodium ions are concentrated in the cytosol of cells (Klionsky *et al*, 1990) while vacuoles are the main compartments of intracellular magnesium and calcium cation storage (Okorokov *et al*, 1980; Cornelius and Nakashima, 1987). The rapid loss of potassium from the cytosol during Cu^{2+} accumulation found in this study and others (Norris and Kelly, 1979) may either be due to a rapid uptake of copper into the cytosol or to copper's effects on the plasma membrane's permeability to potassium. The slower release of magnesium from the vacuole is suggestive of a slower ion exchange which may be explained as a slow accumulation of copper into the vacuole during the copper bioaccumulation process. Calcium release often paralleled that of magnesium rather than that of potassium.

Cobalt accumulation may also result in loss of potassium from the cell but K^+ loss does not always occur during Co^{2+} accumulation (Norris and Kelly, 1977). Addition of Cd^{2+} resulted in a rapid release of approximately 66% of the cellular K^+ (a figure which is similar to that caused by copper in the present study), while Ca^{2+} can inhibit Cd^{2+} uptake and Cd^{2+} induced K^+ release (Norris and Kelly, 1977). In the present study Cu^{2+} appeared to stimulate Ca^{2+} accumulation.

If, as appears from the calculations on copper accumulation by cell walls in chapter 6, the copper accumulated in the initial phase was mostly to the cell wall, then the number of moles of potassium released for each half mole of copper (to keep the Donnan ratio the

same) is often disproportionate. Therefore we can conclude that the loss of potassium from the cell interior only requires relatively small quantities of copper and does not represent a direct exchange mechanism. The fact that so many positive potassium ions are released suggests that the cell interior should become relatively negative, and that this charge gradient may allow for copper accumulation.

7.4.2 Membrane Permeation by Heavy Metals:

In the present study the addition of copper and cadmium (and at much greater concentrations, cobalt) to the cell suspensions caused a large release of potassium ions from the cells. The loss was not stoichiometric but it was extremely rapid. The heavy metal induced potassium loss is therefore probably a result of permeation of the plasma membrane to potassium and possibly other ions.

The research of Oshumi *et al* (1988) suggests that for induction of a turbidity increase in the cell suspensions by Cu^{2+}, copper must be taken up by cells via an energy-dependent transport system, and loss of the barrier function of the plasma membrane occurs when the concentration of Cu^{2+} within the cell reaches a critical value. Cells in the stationary phase were resistant to copper treatment until glucose was added to the cell suspension (Oshumi *et al*, 1988). This turbidity increase could be halted almost instantly by addition of $LaCl_3$ (which blocks the ion ports in the plasma membrane) which suggests that this change took place in individual cells in an all-or-none manner. The specific lesion in the plasma membrane caused by Cu^{2+} therefore requires an energized state of target cells or a membrane potential imposed across the plasma membrane (Oshumi *et al*, 1988).

Cadmium toxicity to cells is believed to be caused by structural lesions in the plasma membrane (Gadd and Mowll, 1983; Joho *et al*, 1985). Cadmium uptake patterns observed indicate that a glucose dependent uptake mechanism of metal accumulation may operate at low concentrations but there is little difference in uptake patterns at higher concentrations with or without glucose. The relationship between Cd^{2+} uptake and K^{+} release is not stoichiometric and it seems likely that the K^{+} release is caused by membrane disruption by the Cd^{2+} binding to organic ligands such as sulphydryl groups. There was also an almost total loss of Mg^{2+} within the first five minutes of contact with cadmium cations (Gadd and Mowll, 1983). This suggests that initial uptake of these cations is *via* a non-selective general uptake system. When a concentration of certain toxic cations is reached within the cell cytoplasm, lesions occur in the plasma membrane. In the case of cadmium it appears that the vacuole membrane may also be affected, which would explain its limited accumulation compared to that of copper cations as observed in the present study.

Mercury, even at low concentrations, may cause irrepairable damage to the plasma membrane of *S. cerevisiae* (Passow and Rothstein, 1960). Mercury (II) chloride causes irreversible damage to the membranes of yeast cells with a subsequent loss of the intracellular potassium and anions. Again this appears to be an all-or-none effect in which individual cells reach their own unique threshold for mercury binding beyond which uncontrolled intracellular ion leakage to the exterior occurs (Passow and Rothstein, 1960). Below this threshold the possibility remains that mercury uptake may cause only a gradual loss

of K^+ from individual cells, although this may in fact be caused by lesion of the cell membrane in only certain cells of the cell population, resulting in massive potassium loss by a few cells. Studies using electron probe x-ray microanalysis have shown that in any population of non-metabolizing yeast cells, cells with partial or near complete K^+ loss coexist in $HgCl_2$-containing cultures, with the latter species becoming increasingly predominant as the mercury concentration increases (Kuypers and Roomans, 1979). The results of that study indicated that the increase in K^+ permeability was associated with the reaction of sulphydryl groups within the cell membrane, but the precise mechanism was not elucidated.

Following treatment of yeast cells with Cu^{2+}, selective leakage of K^+ from the cytosolic compartment appears to create a large concentration gradient of K^+ across the vacuolar membrane and generates an inside negative membrane potential. This in turn may provide a driving force of uptake of positively charged ions into vacuoles (Oshumi *et al*, 1988).

7.4.3 Effect of pH on Copper Bioaccumulation:

The present study shows that the maximum Cu^{2+} accumulation occurred between pH 6 - 9. Low ambient pH values inhibited Cu^{2+} accumulation by yeast cells in suspension. Hydrogen ions may compete directly for facilitated ion transport sites in biological membranes (Schenck *et al*, 1988) and may also compete for intracellular and extracellular binding sites. The lack of accumulation of copper at high pH could be due to a number of factors such as hydroxyl group competition for cations in the solution. Other studies (Huang *et al*, 1988) have indicated that copper accumulation can occur at higher pHs than was the case in the present study, but some of that accumulation was caused by

precipitation of metal hydroxides, while in this study tetramethylammonium hydroxide was used to adjust pH which may prevent formation of hydroxides, or allow precipitation to a lesser extent.

It has been noted that the release of nucleotides, glutamic acid and increased extracellular turbidity caused by copper cations was moderated by pH; pH 6.0 being optimal for release (Oshumi *et al*, 1988). This is close to the optimal copper accumulation pH according to the present study, which strongly suggests that the two activities are linked.

7.4.4 Effect of Temperature on Copper Bioaccumulation:

The present study shows that low temperatures decrease Cu^{2+} bioaccumulation. Likewise release of nucleotides and glutamic acid, and increased extracellular turbidity of yeast suspensions caused by Cu^{2+} bioaccumulation was reduced at temperatures between 0 and 4°C (Oshumi *et al*, 1988).

In another study energy-independent Zn^{2+} binding to *Candida utilis* was negligibly altered over a range of temperatures while in contrast, energy-dependent Zn^{2+} uptake by *C. utilis* did not occur below 5° and above 44.5°C (Failla *et al*, 1976).

7.4.5 Effect of Ionic Strength on Copper Bioaccumulation:

The present study shows that Cu^{2+} bioaccumulation can be reduced to a limited extent by high external concentrations of sodium ions. However, the bioaccumulation of copper was less affected than was the Cu^{2+}-induced release of intracellular cations which was stimulated by the sodium ions.

7.4.6 Ion Inport and Export Mechanisms:

The influx of monovalent cations into the cell is coupled to the activity of the plasma membrane-bound H^+-ATPase, which expels protons, and thereby creates an electrochemical proton gradient across the membrane that has both a chemical component (H^+ ions) and an electrical component (the membrane potential). The membrane potential drives electrophoretic movement of monovalent cations into the cells. The transport is therefore not driven directly by the hydrolysis of ATP, but rather is dependent on the electrochemical gradient that is the result of plasma membrane H^+-ATPase activity. This means that if the transmembrane gradient still exists it is possible for ion accumulation to continue even when the cell is not metabolically active.

Neither *C. utilis* nor *S. cerevisiae* possess a Zn^{2+} efflux system (Failla *et al*, 1976) so they cannot expel this metal once it has entered the cell; the same may be true of most heavy metals. The cells may however have control of the mechanism by which they accumulate specific metals.

The frequently observed stimulation of cation transport across membranes by glucose can be due to two processes. In one process, glucose acts as an energy source for biosynthesis of both cytoplasm and membrane transport proteins. In the second process the H^+ pump is activated which increases the proton gradient, thus stimulating transport of metal ions (the transport and H^+ efflux the gradient are regenerated by a metabolizable substrate via ATP consumption). Both intracelluar and extracellular pH can affect yeast ionic uptake (Jones

and Gadd, 1990). This is to be expected since this would affect the proton (H^+) gradient necessary for cation uptake. An overview of these processes can be seen in figure 7.10.

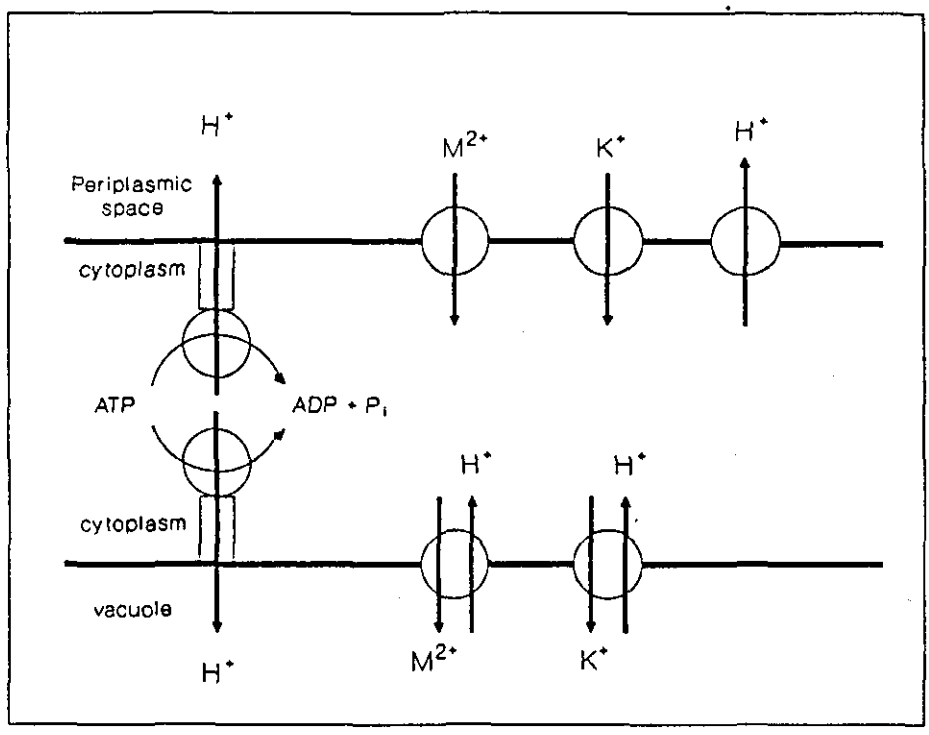


Figure 7.10: *Schematic uptake of metal cations across the cell membranes (after Gadd, 1990b).*

Once the metal in question has entered the cytosol by crossing the plasmalemma, it may be accumulated in the vacuole. The primary mechanism for transport of storage molecules into the vacuole appears to rely on a proton antiport system. The electrochemical potential is capable of driving amino acid and ion transport. ATP hydrolysis is insufficient for this transport process, but must be coupled to the formation of a proton gradient (Klionsky *et al*, 1990).

Apparently an electrochemical potential difference induced by a proton gradient across the vacuolar membrane is a driving force for Ca^{2+} transport into the vacuole (Oshumi and Anruku, 1983). Calcium ions are concentrated in the fungal vacuole by means of an antiport with H^+ or Na^+, or by an H^+-ATPase driven pump (Cornelius and Nakashima, 1987). A Ca^{2+}/H^+ antiport in the vacuolar membrane, indirectly driven by the proton gradient formed by an ATP dependent H^+-ATPase pumping H^+ into the vacuole has been proposed (Eilam *et al*, 1985; Wada *et al*, 1987). Vacuolar pH has been determined to be significantly lower in unenergized stationary cells, while the cytoplasmic pH is slightly higher. However, with the addition of an energy source the pH in the vacuoles of logarithmic and stationary cells are similar (Greenfield *et al*, 1987). Uptake of the calcium ions into the vacuole is optimal at pH 6.4 and obeys saturation kinetics. Calcium transport was shown to require Mg^{2+} ions but was inhibited by Cu^{2+} or Zn^{2+} ions (Oshumi and Anraku, 1983).

Gadd and Jones (1990) noted that there was an increase in the level of Ca^{2+} uptake by the cell when Cd^{2+} was included in the solution, presumably owing to increased permeation of the plasma membrane. Similarly in the present study, Cu^{2+} appeared to increase Ca^{2+} uptake into the cell, although this does not necessarily imply that the calcium was accumulated within the vacuole.

7.5 CONCLUSIONS

From this study and the literature it appears that two major mechanisms are involved in metal bioaccumulation into *S. cerevisiae* cells. The first is the production of a proton gradient across the membranes as a function of H^+-ATPase activity. This gradient allows for ion exchange of cations with H^+ via antiport systems. Although ATPase activity is necessary for this to occur, it is permissible for this activity to precede the uptake of cations and thus a metabolizable substrate is not directly necessary for cation uptake. If the proton gradient already exists, the addition of further metabolic substrates such as glucose can only stimulate metal uptake to a very limited degree, but it may permit metal uptake over an extended duration, provided the metabolic pathways have not been disrupted by heavy metal-induced toxic effects. It would be interesting to observe the effect on yeast heavy metal uptake by the compounds Nigericin and Valinomycin (which selectively effect the pH gradient and the membrane potential, respectively).

The second major mechanism involves permeation of the membranes. The precise effects of this depend on whether the toxic heavy metal in question can then freely enter the cell, which intracellular membranes and organelles are affected if it can, and what intracellular metal chelators are available. The rapid and massive loss of potassium ions caused by this permeation (as found in the present study) may create an internal negative charge which could stimulate metal uptake. The permeation of the membrane may require the cell be metabolically active to and accumulate an initial limited quantity of the heavy

metal in question.

Depending upon the cell molecular contents it may be possible that after the influx of ions into the cell interior, they may be rapidly complexed, ensuring a continued uptake down a free ion concentration gradient (Failla *et al*, 1976).

The release of accumulated intracellular Zn^{2+} from *C. utilis* after treating the cells with organic solvents, or with nyastatin, indicates that some of the metal exists either as free cations or in low molecular mass complexes such as amino acids or nucleotides which would be capable of passing through the cell wall barrier (Failla *et al*, 1976).

As stated above, the internalization of heavy metals may be partially driven by their intracellular chelation. Intracellular chelation is the subject of the next chapter.

8. INTRACELLULAR CHELATORS

8.1 INTRODUCTION

8.1.1 The Binding of Heavy Metal Cations to Proteins:

Intracellular proteins are intimately involved in the binding of heavy metal cations and are often involved in their toxic effects (Blundell and Jenkins, 1977; Hauenstein and McMillin, 1981; Harris, 1991; Baker *et al*, 1987). For instance, a special class of proteins are capable of acting as nucleation sites for inorganic crystals and orientate crystal growth. These acidic proteins and glycoproteins are rich in acidic amino acid residues (specifically aspartate) and often have phosphorylated residues (usually serine), while the polysaccharide moieties also contain charged carboxylate and sulphate groups (Weiner and Addadi, 1991).

Studies of heavy metal-protein interactions conclude that the most important ligands on the protein are the cysteine thiols, cystine disulphides, methionine sulphur, the nitrogens of the histidine imidazole and the side chain carboxylate groups of aspartic and glutamic acid residues (Blundell and Jenkins, 1977; Higaki *et al*, 1992). The binding process may involve the metal ion being bound by several individual protein ligands simultaneously, in a specific three-dimensional arrangement which is related to the possible coordination numbers of the metal and the conformation of the protein (Higaki *et al*, 1992).

Hard ions, such as UO_2^{2+}, usually bind preferentially to hard ligands in proteins such as carboxylates (of glutamate and aspartate), alcohol hydroxyls of threonine and serine, amides of glutamine and asparagine, and phosphate (Blundell and Jenkins, 1977). The hard cations are bound only by proteins acting as multidentate ligands (Blundell and Jenkins, 1977).

The B metals (metals represented in columns of the periodic table between group 8 and the noble gases) such as platinum, gold, cadmium and mercury, bind softer ligands such as thiol (RS), disulphide (R_2S_2), cyanide (CN^-) and imidazole (≡N:) more strongly (Blundell and Jenkins, 1977). Amino groups are intermediate between hard and soft ligands, but these too bind B metals preferentially, as the softer ligands do. Tl^+ and Pb^{2+} ions have non-group valencies and larger radii. Unlike other B metal ions they prefer harder ligands such as carboxylates but may also become oxidized to Tl^{III} and Pb^{IV}, which bind soft ligands more strongly (Blundell and Jenkins, 1977). The mercury cation is particularly reactive towards the negatively charged and polarizable S-groups. The cysteines are less reactive at lower pH when the thiol is protonated. Imidazole groups of histidines are frequently involved in the coordination of zinc atoms (Blundell and Jenkins, 1977).

8.1.2 Metallothionein:

A class of proteins which are rich in cysteine residues and are capable of chelating relatively large quantities of metal are the metallothioneins (Margoshes and Vallee, 1957; Kägi and Schäffer, 1988; Lerch, 1981; Hamer, 1985). The metal chelating-ligands in metallothionein (MT) are a cluster of sulphur atoms of thiols in

the cysteine (cys) residues. Metallothionein preferentially coordinates sets of d^{10} metal ions to form distinct cation clusters. These clusters are the result of specific arrays of reduced cys-thiolate groups which have close proximity to each other, as a function of both the amino acid sequence and the tertiary structure of the protein (Kägi and Schäffer, 1988).

Mammalian MT is a single chain protein and has a molecular mass of 6 000 - 7 000 Daltons (approximately 60 amino acids in length), and about 30% of its amino acid residues are cys; more than one species occurs in mammals and certain other higher animals (Nordberg *et al*, 1972; Lin *et al*, 1990) but the variations are minor. MTs are distinctive in that they contain no aromatic amino acids and therefore do not have the absorption peak at 280 nm which is characteristic of most other proteins (Kägi and Vallee, 1960). They bind 7 equivalents of divalent metal cations (Kägi and Schäffer, 1988). There is a sharing in the metal clusters of the thiolate groups, yielding a measured stoichiometry of nearly three thiolate ligands per divalent metal cation bound in a tetrahedral tetrathiolate coordination; but it is possible that serine and basic amino acid residues are also involved in cation binding (Kojima *et al*, 1976). In mammalian MT there are apparently two topologically separate metal thiolate clusters, $4M^{2+}$-11 cys and $3M^{2+}$-9 cys (Winge and Miklossy, 1982; Neilson and Winge, 1983; Nielsen *et al*, 1985; Kägi and Schäffer, 1988; Boulanger *et al*, 1982).

The clusters act as multidentate metal coordinating-ligands (Kojima *et al*, 1976). The two different clusters show slightly different specificities for certain metals (Nielson and Winge, 1984; Stillman

and Zelazowski, 1989), although at least 18 different ions were shown to bind to the protein, suggesting that there is only limited specificity of binding (Neilson *et al*, 1985). For example MT may bind Zn^{2+}, Cd^{2+} and Au^{2+} (Laib *et al*, 1985). The bound metals may be dissociated at low pH (Kägi and Vallee, 1960, 1961) while oxidation of the MT decreases its metal binding capacity (Geller and Winge, 1982).

Mammalian MTs (class I MTs) are induced by all group Ib and IIb metal ions (Karin *et al*, 1984) and their physiological function is apparently as a reservoir of metal cations for homeostasis of transition metal cations in the cell in a similar manner to a buffer for hydrogen ions (Karin, 1985). The MT may release essential metals to metalloenzymes according to need (Udom and Brady, 1980). Heavy metal chelating proteins other than MT have recently been discovered in primates, and may have similar functions (Waalkes *et al*, 1988).

8.1.3 Fungal Metallothionein:

Yeast MT is a class II MT; that is, it bears little amino acid homology to the mammalian MT (Kägi and Schäffer, 1988), and is also referred to as yeast copper-chelatin or copper thionein. It has been designated a molecular mass of 5 655 daltons (53 amino acids) by sequencing of the gene (Winge *et al*, 1985), although other researchers using sequencing methods suggest a mass of 6 573 daltons (Butt *et al*, 1984; Karin *et al*, 1984). Its metal binding has been well characterised by spectroscopic techniques (Weser *et al*, 1977). It has 12 cys residues, 8 less than mammalian MT, has only one cluster, and may bind up to 4 divalent cations or 8 monovalent cations (Winge et *al*, 1985). Its synthesis is induced by a copper-binding and structurally similar protein which is constitutively produced and encoded by the CUP2 gene

in *S. cerevisiae* (Nakagawa, 1991). Yeast MT binds a wide range of metal cations such as Co^{2+}, Ag^{+}, Cd^{2+}, Cu^{+}, Cu^{2+} and Zn^{2+} (Hartmann and Weser, 1985; Winge *et al*, 1985). Its function in yeast cells appears to be mainly that of detoxification of copper ions (Ecker *et al*, 1986) and up to 60% of cellular copper can be recovered associated with MT (Butt and Ecker, 1987).

Some copper resistant stains of *S. cerevisiae* maintain multiple copies of the yeast MT gene CUP1 even in the absence of selective pressure, but culturing of the yeast in copper-containing solutions does not lead to further amplification of the CUP1 gene (Fogel and Welch, 1982).

Cadmium in cadmium-sensitive *S. cerevisiae* cells was bound almost exclusively to insoluble material such as the cell wall, but in a resistant strain a percentage of the cadmium was found bound to a soluble MT-like protein fraction of molecular weight < 30 000 (Joho *et al*, 1985), which is smaller than the > 70 000 dalton protein detected by Macara (1978) in another strain of cadmium tolerant yeast.

The MT of *Neurospora crassa* differs from mammalian MT in that it is shorter (only 25 amino acids long) and has less cys residues. It is therefore capable of binding less equivalents of metal. The sequence of amino acid residues resembles that of the N-terminal half of mammalian MT, the amino acid sequences being almost homologous (Lerch, 1980). Instead of mammalian MT's two clusters, it has only one (Beltramini and Lerch, 1983) which resembles the ß-domain of mammalian MT (Elgren and Wilcox, 1982).

A third class of MT, phytochelatins, are found in many plant cell cultures and in the fission yeast *Schizosaccharomyces pome* where it has been referred to as cadystin (Kondo *et al*, 1983; Murasugi *et al*, 1984; Grill *et al*, 1985; Grill *et al*, 1987). They are peptides formed of repetitive γ-glutamylcysteine units with a carboxy-terminal glycine, and are 5 - 17 amino acids in length. The involvement of a specific enzyme in the synthesis of this peptide means that it is not a primary gene product. Their synthesis is induced by cadmium, and to a lesser degree by other metals. They can chelate Cd^{2+}, Zn^{2+}, Pb^{2+} and Hg^{2+}, which are found intracellularly almost exclusively chelated with phytochelatin (when present). To date no phytochelatins have been identified in *S. cerevisiae*.

8.1.4 Other Metal Chelating Proteins:

There are numerous metalloproteins and metalloenzymes within the living cell (Vallee and Wacker, 1976), examples of which are given below. If alternative ions are present in excess they may replace the original cation and thereby modify the functioning of the protein (Hauenstein and McMillin, 1981; Verma *et al*, 1982).

(a) Phosphoglycoproteins:

A 12 kDalton phosphoglycoprotein, cadmium-mycophosphatin, has been isolated from the mushroom *Agaricus macrosporus*. It is generally acidic (isoelectric point below pH 2), being rich in glutamate and aspartate residues, and has a number of phosphoserine residues (similar to those proteins referred to at the beginning of this chapter), but contains no sulphur. The carbohydrate moieties include glucose and galactose. It is capable of binding 13.5 mmol Cd^{2+} per mol (Meisch *et al*, 1983).

The cadmium binding, by comparison to similar proteins involved in calcium transport in animals, was assumed to occur at the phosphoserine groups.

(b) Ferritin:

This polysubunit protein is found, in slightly differing forms, in most types of organism. It is capable of binding up to 4 500 atoms of iron. Although iron is the predominant metal stored in ferritin, other divalent and trivalent cations are also bound in smaller quantities. The ability of ferritin to bind metal cations other than iron may be due in part to the incorporation of phosphate anions in the core of the macromolecule. Scatchard plots of binding of such cations as cadmium and zinc by ferritin suggest at least one class of high affinity sites exists for these metals (Sczekan and Joshi, 1989). Ferritin may therefore have a limited role in removal and consequent detoxification of heavy metals.

A ferritin-like molecule has been purified from *S. cerevisiae*, but its iron content was quite low (Raguzzi *et al*, 1988). The protein was 274 kDaltons, composed of 11 kDalton subunits, and contained a haeme group.

(c) Siderophores:

These are not necessarily proteins, as the term includes various low molecular mass iron chelating agents excreted by microorganisms, which are then taken up *via* associated membrane receptors after metal chelation (Chmielowski and Klapcinska, 1986; Messenger and Ratledge, 1985; Hall and Ratledge, 1987). To date there have been no reports of siderophores secreted by *S. cerevisiae*, but the yeast is capable of

reducing extracellular ferric chelates utilizing a membrane bound redox system that is induced in iron-deficient conditions, and thereby accumulating the iron in this manner. Iron is then accumulated in the cell as Fe^{2+} ions as used by ferrochelatase (Lesuisse *et al*, 1990).

(d) Ferrodoxins:

These proteins are similar to MT in that they contain sulphur clusters which are the thiols of cysteine residues; in this case they are used to chelate iron during enzymatic redox reactions (George and George, 1988).

8.1.5 Research Aims:

The aim of this experimental section was to determine whether the strain of *S. cerevisiae* used in the metal tolerance experiments, (see chapter 5) when exposed to copper, had induced the synthesis of a metallothionein or similar metal chelating agent as a function of its resistance to copper toxicity.

8.2 MATERIALS AND METHODS

The copper tolerant yeast cells cultured with copper (see chapter 5) were centrifuged (2 000 x g for 15 min), the pellet was twice resuspended in ultra-pure water before recentrifugation, and then disrupted to obtain intracellular molecular components. The disruption solution consisted of Tris HCl buffer (20 $mmol.dm^{-3}$) containing 1 $mmol.dm^{-3}$ phenylmethylsulphonyl fluoride and 5 $mmol.dm^{-3}$ 2-mercaptoethanol. Phenylmethylsulphonyl fluoride is an inhibitor of serine proteases, while the high copper concentration should have

inhibited most metalloproteases (Boehringer Manneheim). 2-Mercaptoethanol was included to limit oxidation of the sample. Cell disruption was achieved by passing the cells through a pressure cell disruptor (Yeda press, (Linca Lamon Instrumentation Co. Ltd, Tel-Aviv)) thrice at 1 500 p.s.i. The disrupted cells were centrifuged (2 000 x g for 30 min) and the supernatant decanted and subjected to isolation techniques.

Methods of isolation used, such as gel electrophoresis and column chromatography were similar to those of Premakumar *et al* (1975). Sephadex gel-filtration and DEAE Sephadex-A25 chromatography were used to partially purify copper associated material (equipment depicted in Appendix 4) and details of methodology are presented with the respective results. The estimation of the molecular masses of proteins by Sephadex gel-filtration was as described by Andrews (1964). Molecular mass standards used were dextran blue, α-chymotrypsin, cytochrome c, and tryptophan. Gel electrophoresis was performed on a "Mighty small II" electrophoresis unit (Hoffer Scientific Instruments, San Francisco). The stacking gel was 3% acrylamide and 2.7% bis-acrylamide (T = 5.7%, C = 47%), and the resolving gel was 7.5% acrylamide and 2.7% bis-acrylamide (T = 10.2%, C = 26%). Coomassie brilliant blue G250 in perchloric acid solution was used for staining the polyacrylamide gels after electrophoresis as per the method of Reisner *et al* (1975). Protein concentration was followed spectrophotometrically at 280 nm (Appendix 3). The scheme of purification is depicted in figure 8.1.

To investigate whether extracellular copper chelating proteins were present in the extracellular growth medium, ammonium sulphate precipitation of the extracellular medium was undertaken. Extracellular growth medium from the copper tolerant culture was separated from the cellular fraction by centrifugation (5 000 x g for 30 min.) and the pellet discarded. The supernatant (200 cm^3) was maintained at 4°C and 103.2 g of ammonium sulphate (NT chemicals) was systematically added to the solution to obtain a 80% saturation. After 3 hours the solution was centrifuged at 5 000 x g for 20 min. The resultant pellet was resuspended in 10 cm^3 of buffer (20mmol.dm^{-3} Tris HCl buffer pH 7.9/ 5 mmol.dm^{-3} 2-mercaptoethanol) and filtered to remove colloidal material. This solution was then fractionated on a Sephadex G-75 column (details presented with the results).

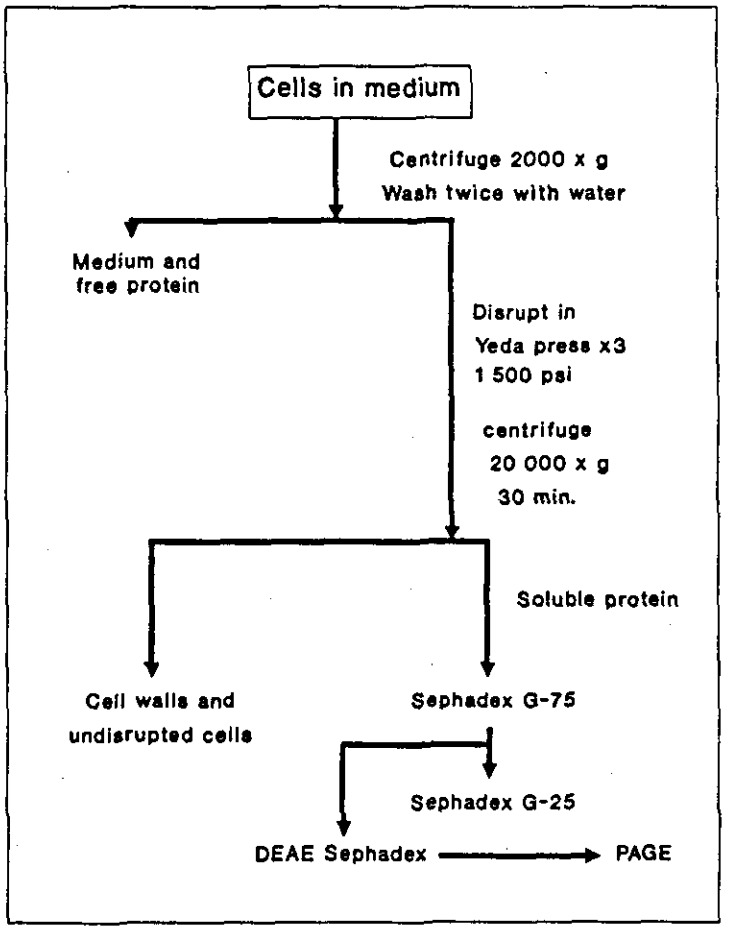


Figure 8.1: Scheme of isolation of intracellular copper associated material.

8.3 RESULTS

The cells were lysed by the pressure cell. The fact that these copper tolerant cells could be easily lysed by this method while far less breakage occurred with the native cells suggests that copper either inhibits wall formation or modifies cell wall structure. The fractionation of a supernatant from a centrifuged sample on Sephadex G-75 yielded only a single copper peak (figure. 8.2). There was also a small zinc peak detected, but iron levels were below the level of detection (0.1 $\mu g.cm^{-3}$).

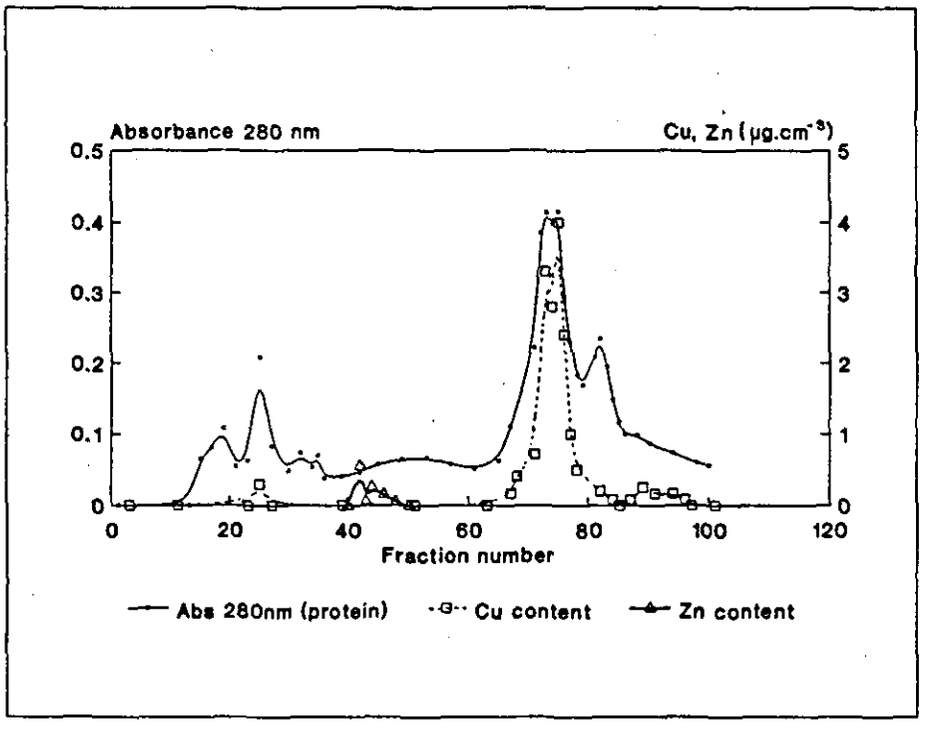


Figure 8.2: Sephadex G-75 chromatography profile of intracellular copper chelating molecules extracted from copper tolerant yeast cells. Buffer: 20 $mmol.dm^{-3}$ Tris/5$mmol.dm^{-3}$ 2-mercaptoethanol. Column height: 75 cm, volume: 340 cm^3. Temperature: 20°C. Flow rate: 0.4 $cm^3.min^{-1}$. Fraction volume: 4 cm^3.

Subsequent anion-exchange chromatography of the combined and freeze-dried copper peak fractions (numbers 72 to 81) yielded a single copper containing peak.

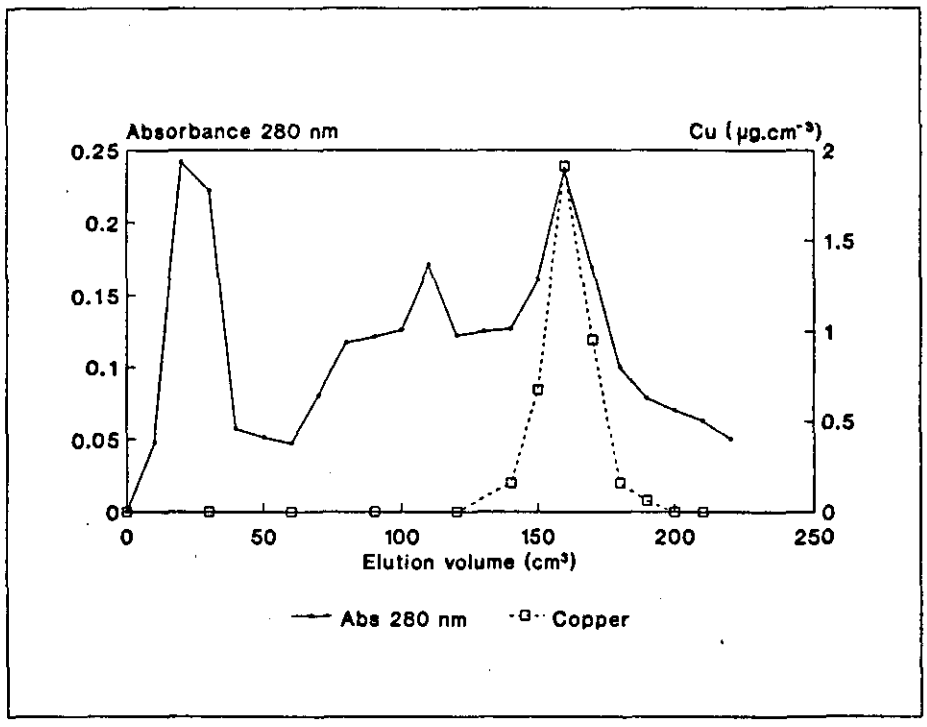


Figure 8.3: DEAE Sephadex A-25 chromatography profile of intracellular copper chelating molecules extracted from copper tolerant yeast cells. Buffer: 20 mmol.dm^{-3} Tris/5mmol.dm^{-3} 2-mercaptoethanol. Column height: 13 cm, volume: 10.2 cm^3. Temperature: 20°C. Flow rate: 0.6 cm^3.min^{-1}. Fraction volume: 5 cm^3. Eluted with 0 - 0.4 mol.dm^{-3} NaCl.

To determine if the copper-containing compound was a type of siderophore, the extracellular culture medium was investigated for large quantities of this compound. Ammonium sulphate precipitation of the extracellular culture medium yielded a similar profile to that of the intracellular solution when fractionated on a gel-filtration chromatography column (figure 8.4). However, the low concentration of this compound in the extracellular medium suggests that it is

primarily an intracellular compound and therefore cannot be considered to be a siderophore.

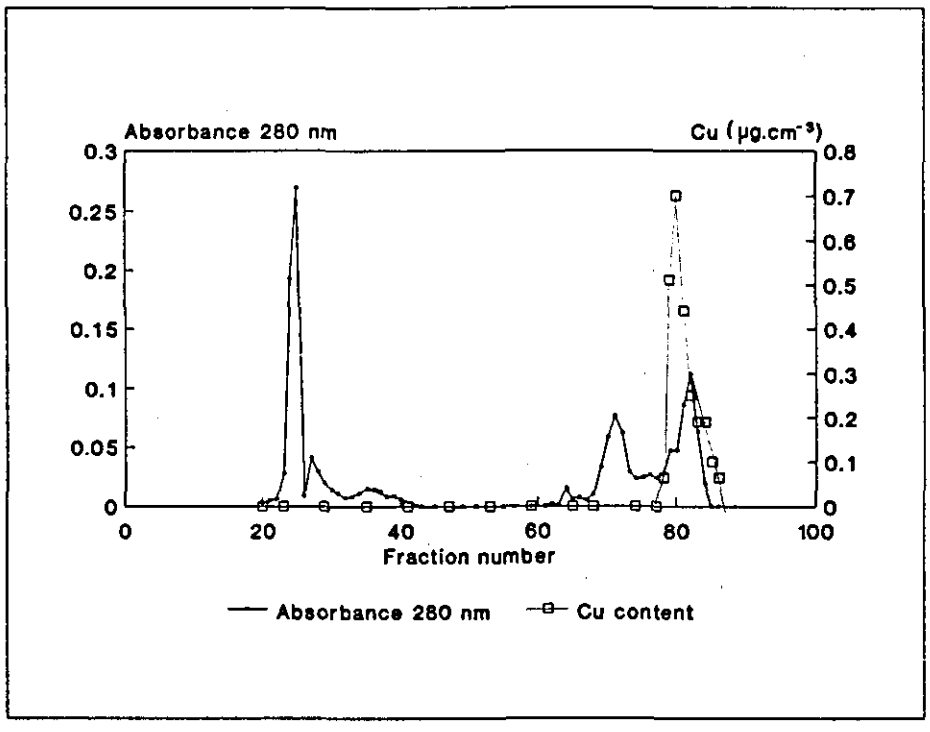


Figure 8.4: Sephadex G-75 chromatography profile of extracellular copper chelating molecules. Experimental conditions were identical to those in figure 8.2.

The subsequent gel exclusion chromatography on Sephadex G-25 of the combined and freeze-dried copper peak fractions (numbers 72 to 81) from the Sephadex G-75 chromatography of the intracellular proteins (from figure 8.2) revealed that the metal binding molecule was of a low molecular mass (figures 8.5 and 8.6).

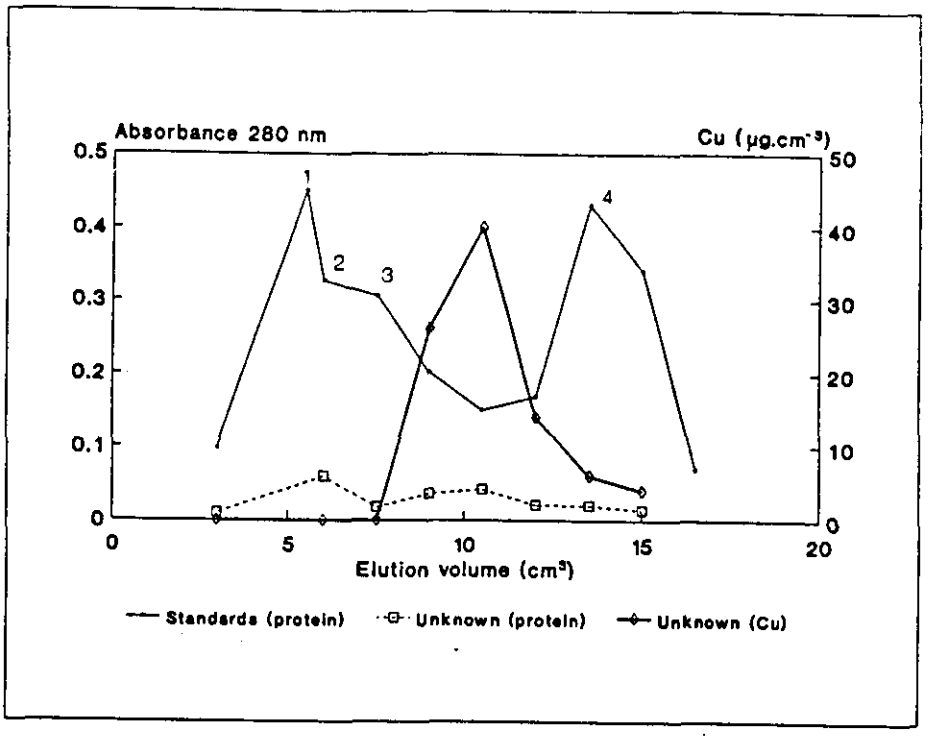


Figure 8.5: Sephadex G-25 chromatography of intracellular copper chelator from yeast cells. The standards (run separately) were: (1) Dextran blue, (2) α-chymotrypsin, (3) cytochrome c, (4) tryptophan. Column height: 18 cm, volume: 14 cm^3. Flow rate 10 $cm^3.hr^{-1}$.

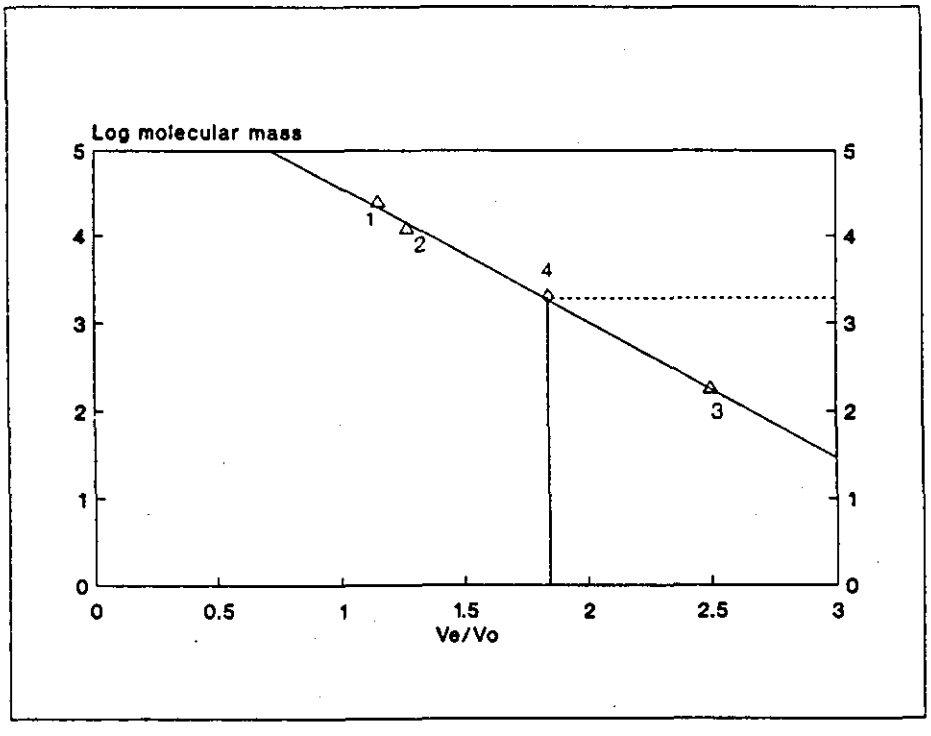


Figure 8.6: Linear log representation of molecular mass standards after gel exclusion chromatography (Sephadex G-25). Standards (1) α-chymotrypsin, (2) cytochrome c, (3) tryptophan; (4) is the copper containing protein.

The molecular mass of the copper binding material was approximately 2 000 daltons (derived from figure 8.6), which is too low to be yeast MT, and is even less than the molecular mass of MT from *Neurospora crassa*.

Analysis the copper peak from the anion-exchange chromatography column by polyacylamide electrophoresis (PAGE) yielded only a single band (figure 8.7), therefore suggesting that it was a relatively pure preparation.

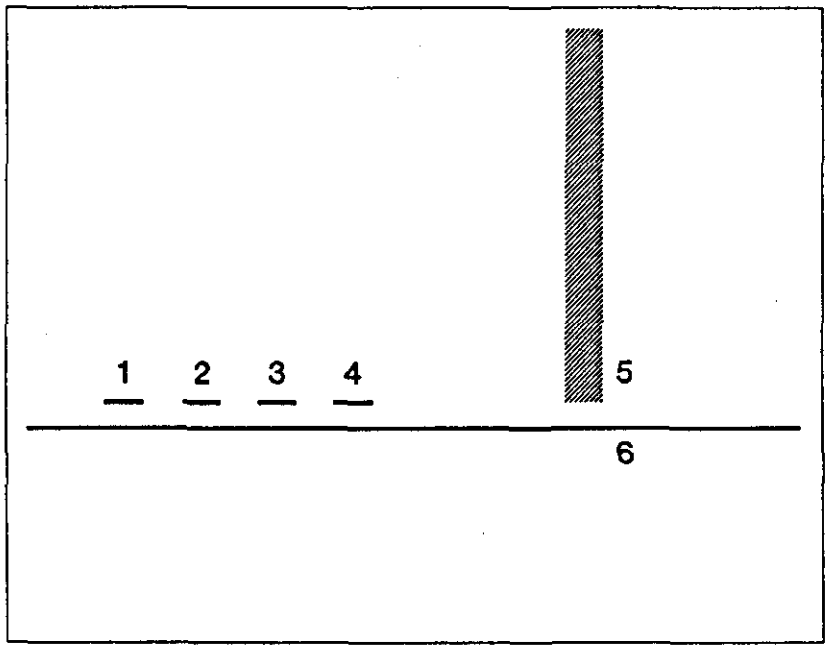


Figure 8.7: PAGE gel electrophoresis of fractions 75 - 80 from the Sephadex G-75 column run of an intracellular copper chelator. Lanes 1, 2, 3, and 4 are bands resolved from the probably purified copper chelating material. Lane 5 is a crude intracellular solution. The horizontal line (6) represents the sample front as indicated by bromophenol blue.

A characteristic of MT is its high molar equivalent capacity for copper ions. The ratio of copper ions to their molecular chelators from the major copper-containing peak in figure 8.6 was calculated as follows: The number of copper ions per protein molecule can be calculated from the copper concentration: $2\mu g.cm^{-3}$ (fraction 4 from the Sephadex G-25 column, figure 8.5).

The number of moles of Cu = mass of metal/atomic mass,

$= 2 \times 10^{-6}$ g/ 63.546 $g.mol^{-1}$

$= 3.2 \times 10^{-8}$ mol

= 32 nmol

Protein: Absorbance of the protein is 0.18 at 280 nm, which from the standard curve (Appendix 3) gives 0.3 $mg.cm^{-3}$.

The molecular mass of the protein is approximately 2 000 daltons.

The number of moles $= 0.3 \times 10^{-3}$ g/2 000 $g.mol^{-1}$

$= 1.5 \times 10^{-7}$ mol

= 150 nmol

Therefore Cu^{2+}/protein mol/mol = 32/150

= 0.21 $mol.mol^{-1}$.

This clearly indicates that the molecule is not a MT type molecule. Another characteristic of MT is its low ultra-violet absorption at 280 nm. The absorption spectrum of the copper chelating compound was determined (figure 8.8), showing a pronounced peak in the 270 - 310 nm region.

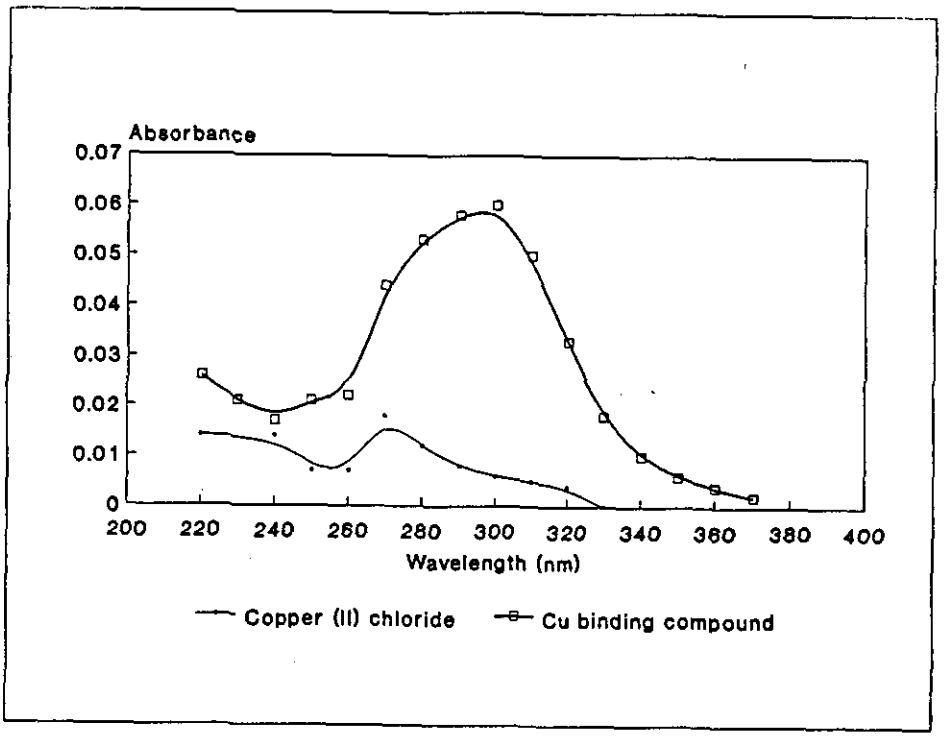


Figure 8.8: The absorption spectrum of the copper binding compound from Sephadex G-25 chromatography.

8.4 DISCUSSION

In the present study no intracellular MT type protein was detected in the strain of *S. cerevisiae* investigated. The major copper binding component was a small molecule of approximately 2 000 daltons that may be a short peptide of about 20 amino acids. Although it could be a proteolytic product of MT, there are a number of reasons why this is unlikely. First the peptide, unlike MT, absorbs light strongly at 270 - 300 nm; secondly, MT of *S. cerevisiae* is thought to be resistant to proteolysis when it has bound its quota of Cu^{2+} (Weser *et al*, 1986); and thirdly, the molecules found in the present study accumulated at most only one copper atom per molecule. There is indirect proof that the copper was bound to this molecule since free Cu^{2+} cations bind to

Sephadex G-75 whereas the detected copper from the cytosol of copper tolerant yeast did not.

In certain *S. cerevisiae* strains MT is synthesised *de novo* as a function of copper stimulated induction, while Cd, Zn or Hg do not stimulate MT induction (Premakumar, 1975). A cadmium-binding protein isolated from a cadmium-resistant strain of *S. cerevisiae* was found to be similar in amino acid composition to MT, but the strain of yeast showed no co-resistance to Cu^{2+}, Zn^{2+} or Hg^{2+}, indicating that it was specifically induced by cadmium and not other heavy metals (Inouhe *et al*, 1989). This is important in that even if a strain of waste yeast being utilized produced a metallothionein it would only be induced by one specific metal. If this particular metal was in low levels in the wastewater, MT would not be synthesized and would therefore not be involved in metal bioaccumulation.

The massive excess of bound copper found in the cells compared to the quantity of bound zinc (figure 8.2) indicates that the Cu^{2+} binding compound may have been induced by the copper.

Karin *et al* (1984) have suggested that growth in copper brewing vats, of *S. cerevisiae* strains, from which laboratory strains were produced, may have provided the environmental conditions for selection of copper-resistant strains

Many functions and methods of utilizing MT from yeast have been advanced (Butt and Ecker, 1987; Marx, 1989), such as altered amino acid sequences for metal specificity enhancement, and modification and exporting the MT to the yeast periplasmic space where it could be more

effectively used for metal accumulation and retrieval, although these involve genetic engineering.

Synthetic MT-like oligopeptides containing cysteine residues have been shown to bind and detoxify metal cations in rodents, although the affinity of the oligopeptides for the cations was a few orders of magnitude lower than that of MT (Yoshida *et al*, 1979). A metal chelating resin has been developed which mimics the action of MT, however the present method of synthesis is prohibitively expensive for industrial purposes (Yin and Blanch, 1989).

Metallothionein genes could be introduced into yeasts which lack the gene (such as the strain used in this study). The MT of *Neurospora crassa* has been chemically synthesised, inserted into a plasmid and expressed in the bacterium *Escherichia coli* (Sugimoto *et al*, 1988), while mammalian MT has been inserted into and been shown to be functional in yeast (Thiele *et al*, 1986), so there is no reason why MT genes should not be implanted into any yeast desired. However the purpose of the present study is to show how waste yeast may be utilized for metal accumulation with minimal extra expense, and unless the CUP1 gene could be inserted into a plasmid and easily spread amongst yeast, this option would be prohibitively expensive.

In conclusion, a Cu^{2+} chelating compound was partially isolated from a Cu^{2+} tolerant strain of *S. cerevisiae*. This low molecular mass compound is believed not to be a MT due to its low molecular mass, low capacity for Cu^{2+}, and its maximal absorbance of light at 260 - 320 nm. The high levels of copper within the cell as compared to intracellular zinc levels signify, however, that extracellular copper has not been

totally excluded by the cell and this compound is involved in the coordination of free copper within the cell.

There is a link between decreased accumulation of Cu^{2+} and the lack of MT in this strain. A cell which takes up copper and does not chelate that metal intracellularly using a MT type compound could exhibit symptoms of copper toxicity. Therefore it may be assumed that any Cu^{2+} tolerant yeast which is able to control intracellular concentrations is unlikely to produce a MT. This suggests that the Cu^{2+} tolerance exhibited by this strain of yeast was mediated primarily by the cell membrane.

The Cu^{2+} binding compound found in the present study was present in only relatively low concentrations in the extracellular medium, suggesting that this compound is not a siderophore.

PART 3: BIOSORPTION

9. BIOSORPTION OF HEAVY METAL CATIONS BY NON-VIABLE YEAST

9.1 INTRODUCTION

Biosorption is the accumulation of metals without active uptake (Volesky, 1987). Biosorption can be considered as a collective term for a number of passive accumulation processes which in any particular case may include ion exchange, coordination, complexion, chelation, adsorption and microprecipitation. It may occur even when the cell is metabolically inactive, such as when it has been killed by chemical or physical means.

Cell wall binding of metals may also be regarded as biosorption of metals (see chapter 6), but this chapter is concerned with the biosorption of metal ions to non-viable yeast biomass.

The advantages of non-viable cells are numerous. Killed cells may be stored or used for extended periods at room temperature without putrefication occurring. Moreover living cells are prone to the toxic effects of effluents, which may result in cell death, thereby negating any of the advantages of utilizing live cells. Biosorption by non-viable organisms would be ideal for use in these conditions (Darnall *et al*, 1986).

Some methods of killing cells may actually improve biosorption properties of the biomass (Brierley *et al*, 1986). Some killing methods, such as immersion in formaldehyde, would cross link the cells thereby

simultaneously immobilizing them. Non-viable *Rhizopus arrhizus* biomass, killed by 1% formaldehyde solutions has proved to be extremely durable (Ileri *et al*, 1990) and is therefore able to endure more chemically aggressive environments than living biomass. Non-viable microorganisms also represent a reduced health hazard when utilizing potentially pathogenic strains. Finally, processes using inactive or killed microoganisms have the advantage of not depending on a supply of nutrients for cell growth, nor do they require a starting-up time for the necessary biomass bulk to grow (Bordons and Jofre, 1987).

Isolated cell walls of yeasts have been shown to be capable of accumulating metal cations (see chapter 6), as are individual molecular components of cell walls (see chapter 6); both of these means of metal binding would be available in killed cells. With killed cells, however, vacuolar compartmentalization or induction and synthesis of metal chelating proteins would not occur.

S. cerevisiae, when killed by heat drying and grinding actually increased its ability to accumulate a range of metal cations, including uranium (Kuyucak and Volesky, 1988); however in other experiments a decrease in uptake has occurred after killing, such as in certain biosorption experiments with *R. arrhizus* (Tobin *et al*, 1990), where chemical killing methods reduced uptake by up to 60%. *Rhizopus* biomass could be killed and utilized in fixed bed columns to recover metals (Treen-Sears *et al*, 1984). Non-viable *R. arrhizus* has been shown to accumulate uranium (Tsezos and Volesky, 1982a), and thorium (Tsezos and Volesky, 1982b). Chemical modification may also change the specificity of biosorption (Nakajima *et al*, 1981).

BIOSORPTION BY NON-VIABLE YEAST

A company based in the U.S.A., Advanced Mineral Technologies Inc., has developed a metal-accumulating non-living biomass derived from microorganisms (type unspecified). The biomass is prepared by exposure of microorganisms to caustic conditions at elevated temperatures, and the material is then dried and ground to a granular consistency to increase the surface area available for metal binding. This proprietary product, referred to as metal recovery agent (MRA), is capable of accumulating large quantities of metal cations and is more than 99% efficient in metal cation removal from dilute metal solutions (Brierley *et al*, 1985, 1986; Brierley 1987). The agent can accumulate a wide range of metal cations including gold, silver, palladium, platinum, lead, copper, cadmium and zinc.

The material is selective in that it will bind certain heavy metals in preference to others and also in preference to alkali or alkaline-earth metal cations such as calcium, potassium, sodium or magnesium (Brierley *et al*, 1986). It has been successfully used in pilot scale trials (Brierly, 1987).

MRA functions in a pH range of 2 - 11 and maximally in the range pH 4 - 8. The type of cation salt can affect biosorption. For example, MRA is capable of accumulating gold from gold-cyanide complexes but with reduced total uptake as compared to other salts of gold (Brierley, 1987).

Caustic treatment has the advantages that it destroys autolytic enzymes that cause putrefication of biomass and *inter alia* removes lipids and proteins that mask reactive sites (Brierley *et al*, 1985;

McGahren *et al*, 1984; Muraleedharan and Venkobachar, 1990). Other researchers have utilized alkali treatment to produce metal ion-accumulating non-living biomass. A proprietary method has been developed for producing a fungal hyphae-associated fibrous textile filter which is treated with alkali to expose chitin and chitosan found in the hyphal walls. When these filters are assembled in a column they can be used to accumulate metal ions from industrial wastes (Sagar *et al*, 1988). Brierley *et al* (1986) noted that for some species the caustic treated biomass had superior metal ion accumulating properties compared to the native biomass, although some species showed the reverse effect. Kuyucak and Volesky (1989) found that treatment with warm dilute alkali greatly reduced the ability of certain biomass to accumulate cobalt, while others have found no effect (Huang *et al*, 1988).

Each biosorbent has a unique set of metal cation binding properties. Yeast cells killed by extreme chemical and physical conditions may also possess very different metal accumulating parameters compared to the original yeast. The aim of this section of this study was to determine some of the parameters of metal biosorption by a granular biomass which was produced by heating yeast in alkaline conditions.

9.2 MATERIALS AND METHODS

9.2.1 Preparation of the granular biosorbent:

This was prepared by mixing 100 cm^3 wet mass of yeast with an equal volume of 2 $mol.dm^{-3}$ NaOH and heating the resultant solution to 70 - 90°C for 15 min. The product was filtered through Whatman No. 1

filter paper, washed with deionized water, and refiltered. The product was dried on grease-proof paper at 70°C for 12 hours and then peeled off, milled to a gritty consistency, and passed through a mesh (500 microns, 30 mesh).

The hot alkali-treated yeast granules were visualized using scanning electron microscopy (figures 9.1 - 9.3). At low magnification the granules appear to have a rough surface (figure 9.1). At higher magnification (figure 9.2) it becomes obvious that the material has an extensive surface area available for metal adsorption. At yet higher magnification (figure 9.3) flakes of material can be observed which could provide a large surface area for cation adsorption.

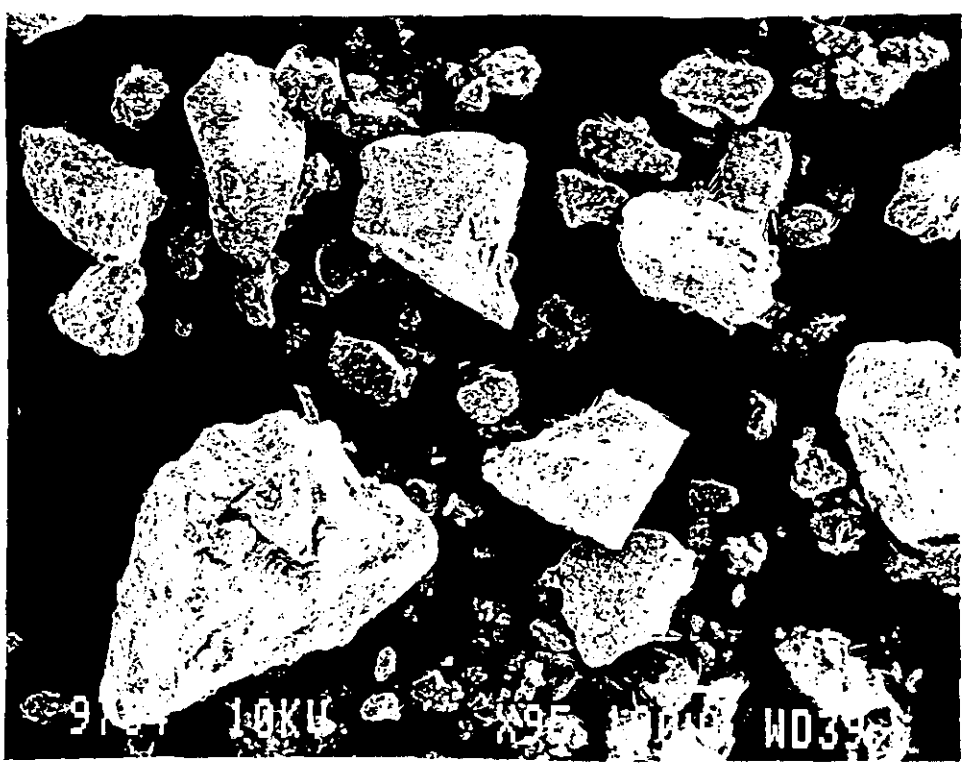


Figure 9.1: *Scanning electron micrograph of grains of dried yeast biomass that had been heated in 2 mol.dm^{-3} sodium hydroxide. Magnification x 95.*

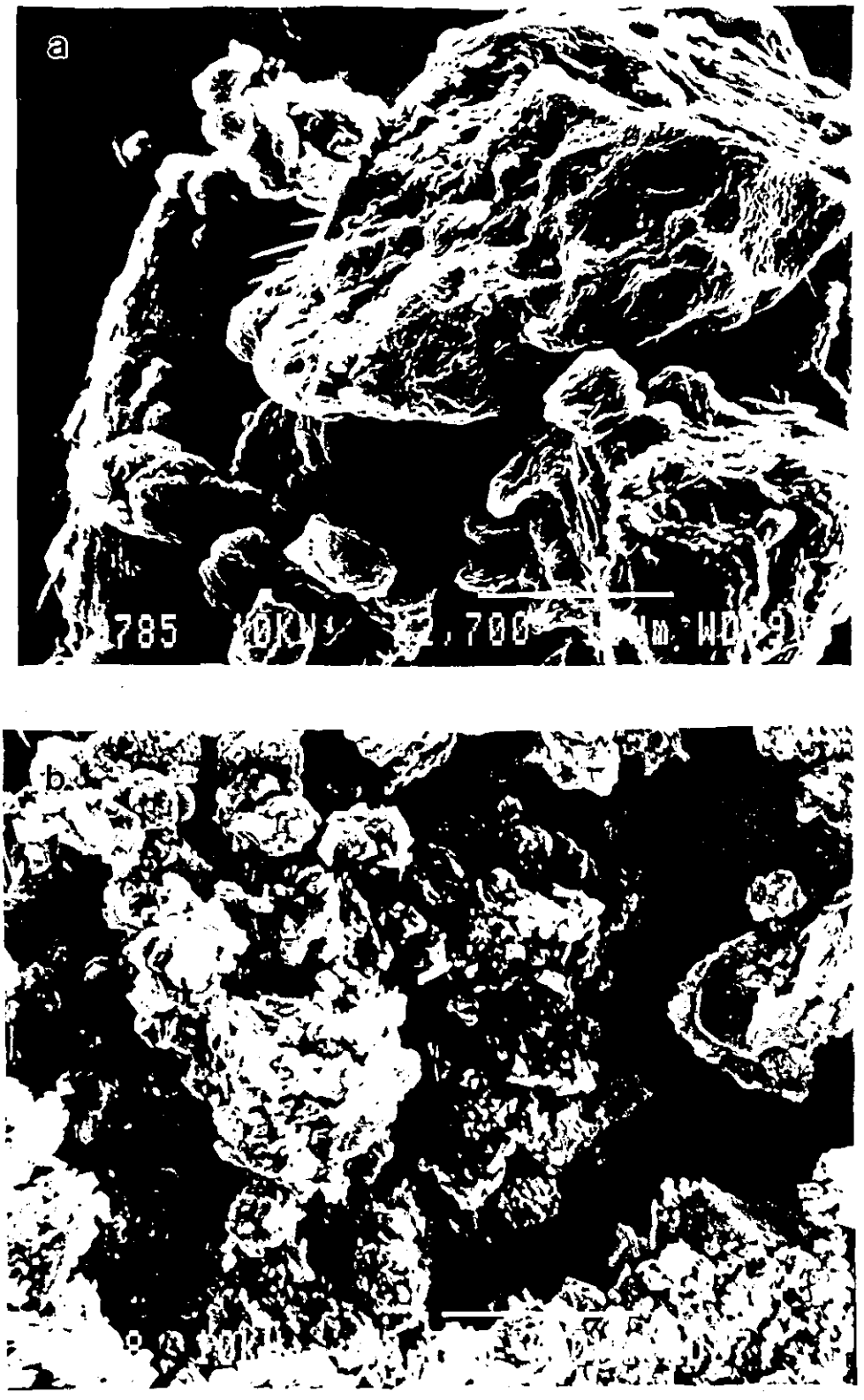


Figure 9.2: Scanning electron micrographs of the surface of grains of granular biosorbent, x 2 700 magnification ((a) and (b)). Note the large surface area.

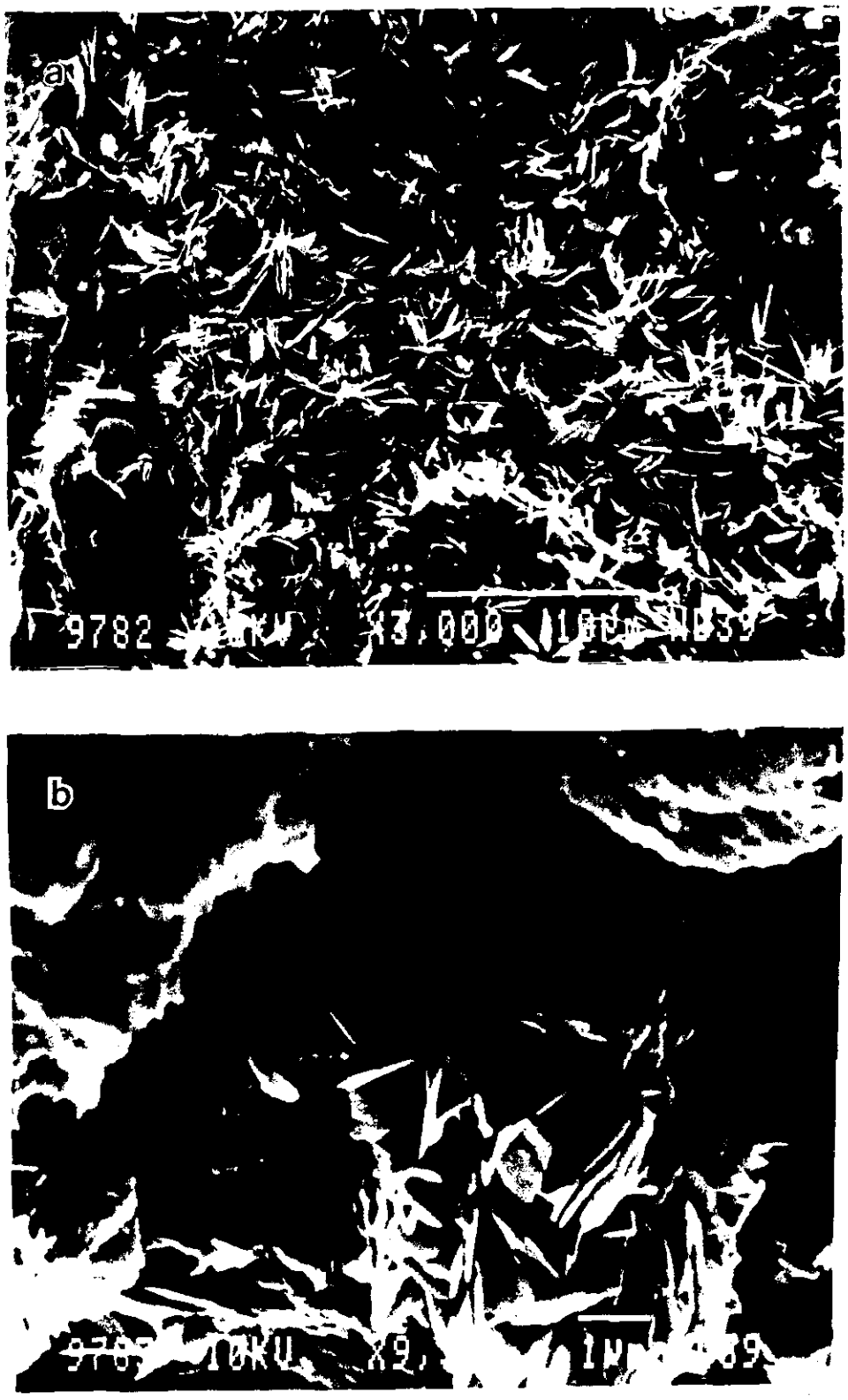


Figure 9.3: High magnification scanning electron micrographs of the surface of grains of granular biosorbent, x 3 000 (a) and 9 500 (b) magnification. Note the crystaline flakes which supply a very large surface area.

9.2.2 Equipment:

Chromatography equipment: Eyela MP-3 Peristaltic pump, Rikakikai Co. Ltd. Tokyo; 2112 Redirac fraction collector (LKB); LKB column system. Spectrophotometric equipment: Bausch and Lamb Spectronic 1001; Varian Techtron 1000 Atomic Absorption spectrophotometer.
Miscellaneous equipment: pH meter 761 Calimatic (Knick); laboratory oven. Spectrapor dialysis tubing (6 - 8 kDalton cut-off, Spectrum Medical Industries Inc., Los Angeles). Milli-Q Millipore ultra-pure (18 ohm) water was used throughout these experiments.

9.2.3 Experimental conditions:

Continuous flow biosorption by columns of granular biosorbent:
Chromatography columns were loaded with aqueous slurries of granular biosorbent (5 g), and metal solutions at 100 ppm (except Ag^{+} (50 ppm) and Hg^{2+} (200 ppm)) were pumped upwards through the column at a flow rate of 24 $cm^3.hr^{-1}$. The ambient temperature during these biosorption experiments was 20°C. The column volume was 35 - 45 cm^3. Biosorption was indirectly monitored by measuring the metal concentration in the effluent. Typical experimental results are presented rather than averages of repeats. Concentrations of metals are applied as parts per million (ppm), i.e. mg of metal per dm^3, a unit of concentration that is commonly used when reporting conditions in wastewater treatment processes.

Batch biosorption of mixed cationic solutions:
Direct comparison of the preference of the granular biosorbent for binding specific metals was attained using a dialysis system and equimolar concentrations of metals. The granular biosorbent (1 g) was

washed thrice with 50 cm^3 of ultrapure water and then placed in dialysis tubing (15 cm^3 internal volume). The tubing was placed in 0.5 dm^3 of metal contaminated solution. The solution contained a mixture of equimolar metal salts (1 $mmol.dm^{-3}$) with a common anion. Two parallel experiments were conducted where the sulphate salts of Cr^{3+}, Cu^{2+}, Cd^{2+} (group 1) and the nitrate salts of Pb^{2+}, Cu^{2+}, Ni^{2+} (group 2) were separately incubated with the granular biosorbent for 60 hours. The dialysis tubing was then washed for 2 hours against two changes of 100 cm^3 of water.

The granular biosorbent was then removed from the dialysis tubing and digested at 100°C with 20 cm^3 of 55% nitric acid (AECI, analytical grade) and an equal volume of water. The resultant solution was then assayed for the concentrations of the various metals.

Rate of metal biosorption by granular biosorbent.

To 180 cm^3 of water was added 1 g of granular biosorbent and 20 cm^3 of 1 000 ppm metal solution of either Cu or Cr salts or 2 000 ppm of Pb salts (lead has a significantly higher atomic mass that the other two metals and addition of greater metal mass approximately compensates for this). Upon metal addition the reaction vessel was shaken vigorously and a sample drawn and immediately filtered. During the progress of the experiment aliquots of the solution were periodically withdrawn and immediately filtered. The filtrates were assayed for metal content.

9.3 RESULTS

Biomass, treated in various manners, was dialysed against a copper solution and the copper accumulation capacity compared. The granular biomass produced from yeast by treatment with hot alkali reduced the quantity of Cu^{2+} binding to the biomass as compared to the bioaccumulation by the viable (native) yeast by 27% (figure 9.4). However,the biomass treated with hot alkali (not granularized by filtration, washing and drying, but rather subjected to extensive dialysis against water) accumulated 117% more copper than the native cells and 200% more than the granular biosorbent. The hot alkali treatment removes a considerable amount of manno-protein from the cell (see section 6.4) and this would not be retained during normal filtration and washing. This means that the granular biosorbent is composed primarily of glucan and some chitosan (most of the chitin would have been converted to chitosan by the hot alkali procedure). The alkali soluble manno-protein produced by the hot alkali treatment could be precipitated with $CuCl_2$, or less effectively with $CaCl_2$. Its high capacity for copper suggests that the manno-proteins could be used as heavy metal flocculants. The granular biosorbent has the advantage, however, of being macroscopic in size and therefore can be retained by a simple mesh without the requirement of expensive membrane filters or immobilization. For this reason the heavy metal biosorption by the granular material was studied further.

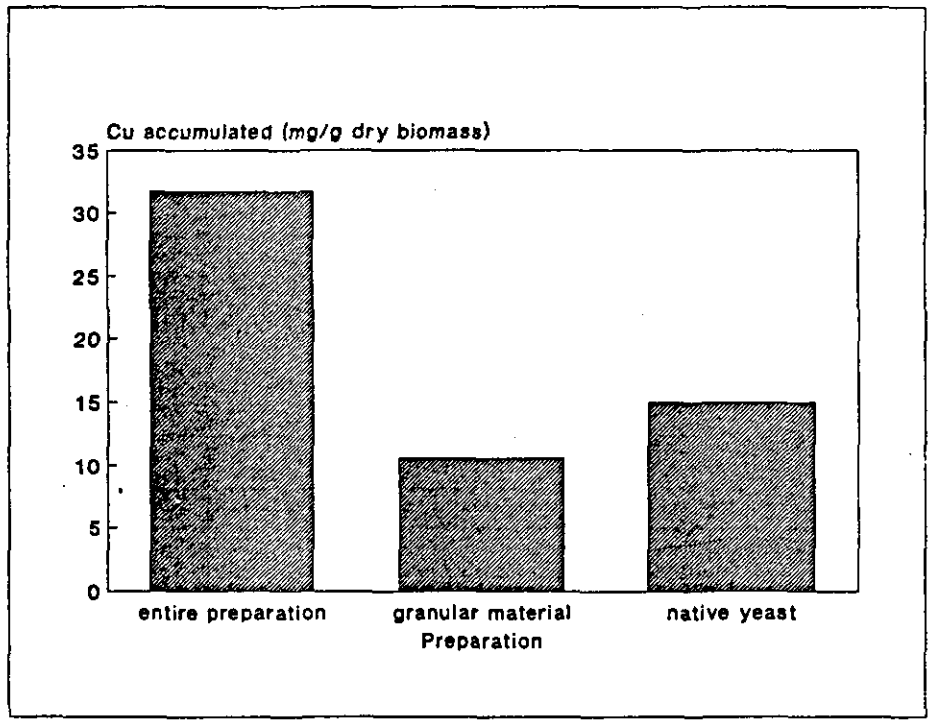


Figure 9.4: Copper (100 ppm as $CuCl_2$) accumulation by different fractions of alkali treated yeast compared to native (untreated) yeast cells. The preparations were retained using dialysis membranes.

Passing copper-contaminated aqueous solutions though a column of the granular biosorbent effected complete removal of the copper (figure 9.5). Similar to the living biomass immobilized in columns (see chapter 4) the existence of numerous theoretical plates of equilibrium is responsible for this phenomenon. Even after breakthrough of the copper from the column after 400 cm^3 of influent had been processed, the column was still capable of accumulating copper, although at a lower efficiency, until it was completely saturated. Copper biosorption could be visualized in progress as the biosorbent acquired a green hue which began at the inlet and progresses to the outlet.

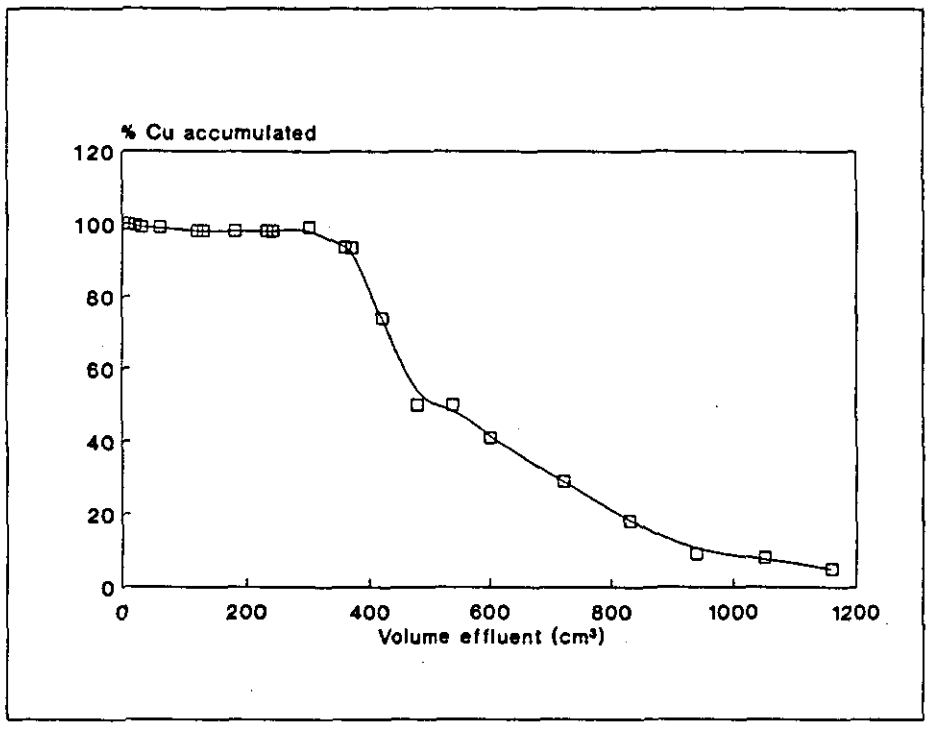


Figure 9.5: Biosorption of 100 ppm copper (as $CuSO_4$) solution by a column of 5 g of granular biosorbent. The influent pH was 5.0.

Moderate variations in pH did not appear to affect the metal binding capacity of the granular biosorbent to a notable degree, as shown by the binding of nickel at pH 7.5 and 6.1 (figure 9.6).

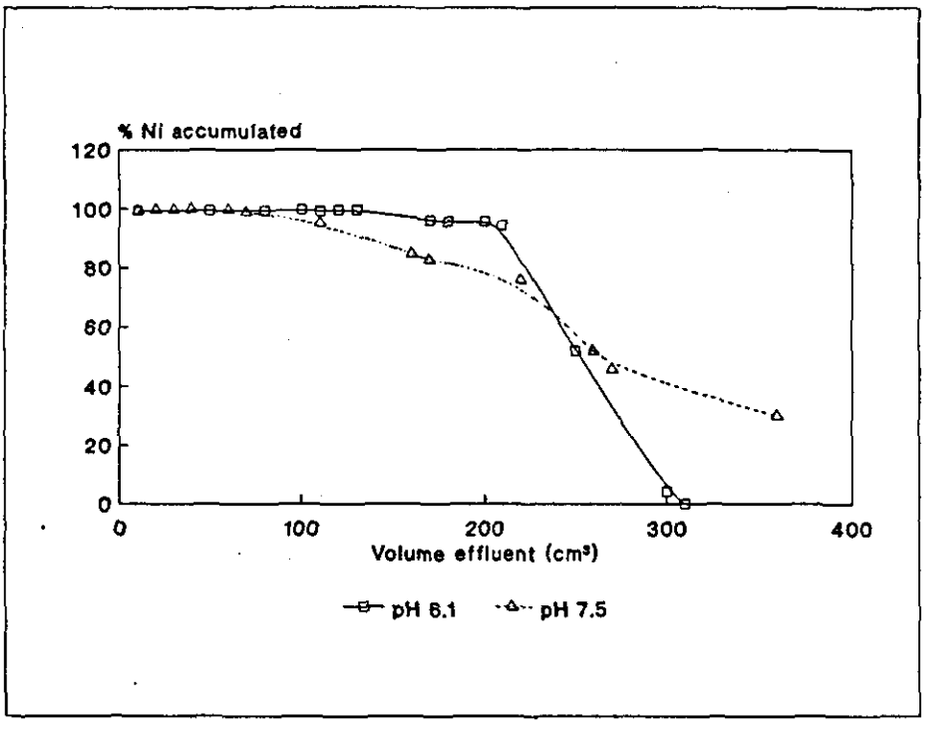


Figure 9.6: Biosorption of nickel by granular biosorbent. The influent pH was 6.1, or modified to pH 7.5 by addition of 50 mmol.dm^{-3} Tris buffer.

Biosorption of iron imparted an orange hue to the biosorbent. A change in the valency of a particular ion appears to affect the metal binding capabilities of the granular biosorbent to a large degree. For example ferric iron was bound to a far greater extent than ferrous iron (figure 9.7). Limited binding was seen as the formation of an orange hue at the inlet, suggesting that this was ferric iron produced by the spontaneous oxidation of ferrous iron in solution. Limited binding of ferric iron should have produced an even colour over the entire column if at all.

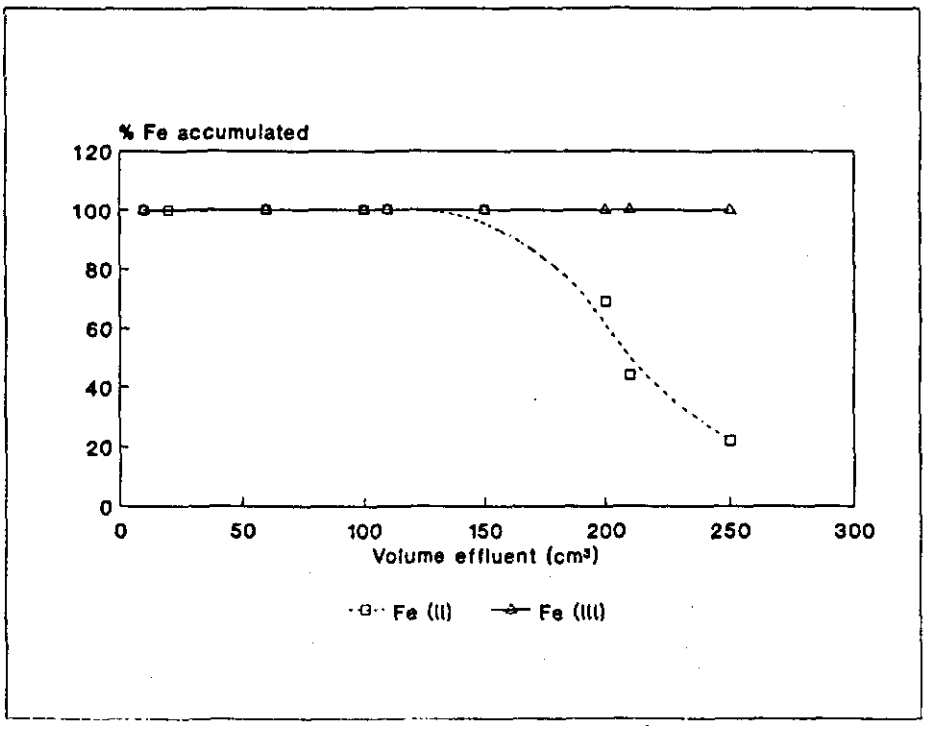


Figure 9.7: Bioaccumulation of ferrous iron (Fe(II) as $FeSO_4$) or ferric iron (Fe(III) as $FeCl_3$) by granular biosorbent, influent pHs were 2.4 pH 3.5 respectively.

The biosorption of Co^{2+} and Cd^{2+}, Pb^{2+} and Hg^{2+}, and Ag^{+} by columns of granular biosorbent are presented in figures 9.8, 9.9, and 9.10. During biosorption of Ag^{+} the granular biosorbent slowly turned dark brown-black as the silver nitrate reacted with the biosorbent due to exposure to light passing through the glass walls of the chromatography column.

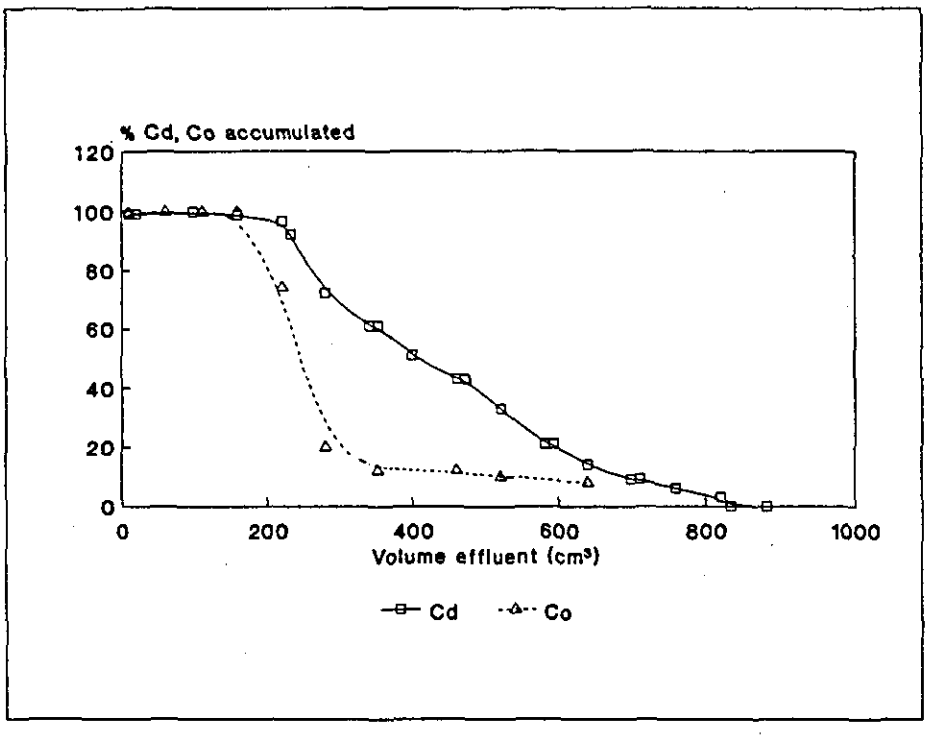


Figure 9.8: Comparison of Cobalt (as $CoCl_2$) and Cadmium (as $CdSO_4$) biosorption by granular biosorbent, influent pH was 3.7 in each example.

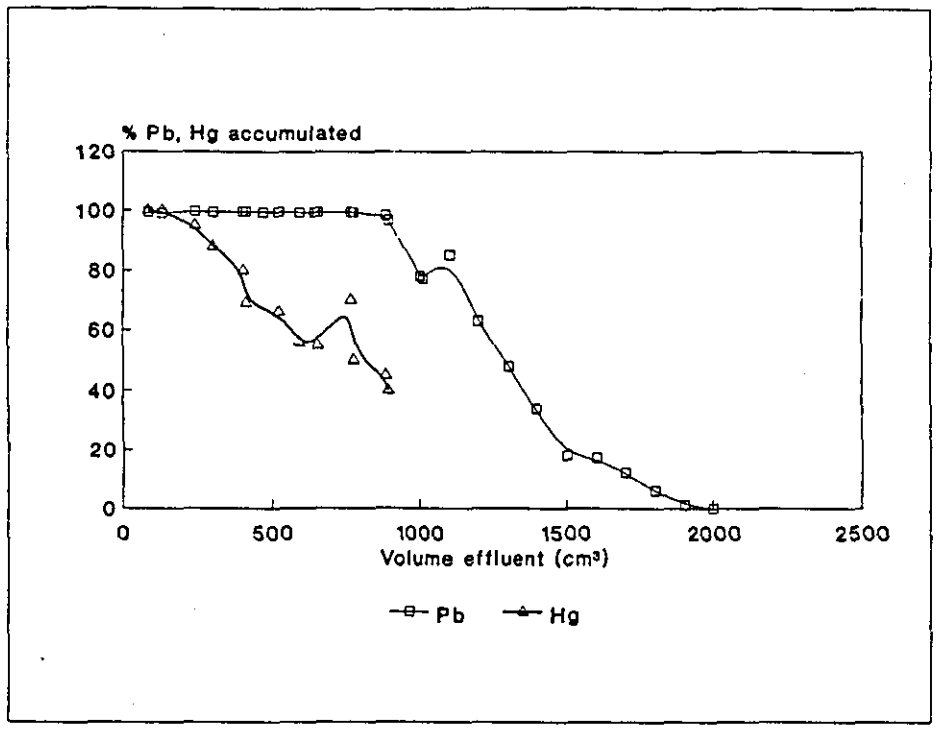


Figure 9.9: Comparison of lead biosorption (100 ppm as $Pb(NO_3)_2$) and mercury (200 ppm as $HgCl_2$) biosorption by granular biosorbent, influent pH was 4.5 and 5.6 respectively.

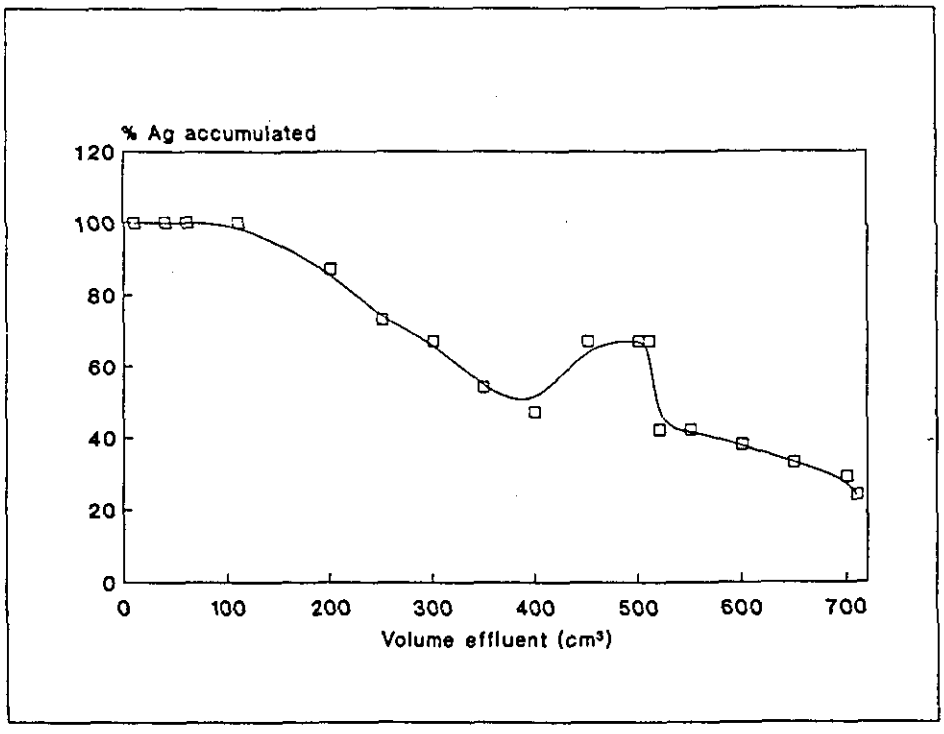


Figure 9.10: Biosorption of silver (50 ppm influent as $AgNO_3$) by granular biosorbent. Influent pH was 5.0.

Chromium (Cr(III)), a trivalent metal cation is bound more effectively by columns of granular biosorbent than the oxidized form of chromium, dichromate (Cr (VI)) which is a divalent anion (figure 9.11), showing that the biosorbent only binds cations effectively.

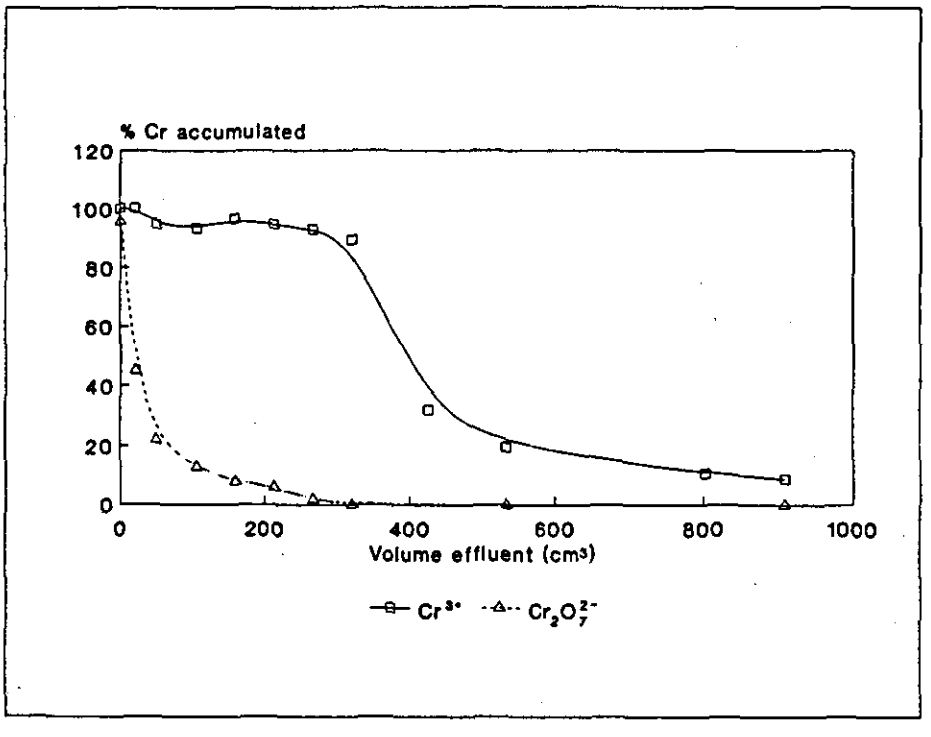


Figure 9.11: Comparison of Cr (III) (100 ppm as $Cr_2(SO_4)_3$) and dichromate (VI) (100 ppm as $K_2Cr_2O_7$) biosorption by granular biosorbent. Influent pHs were 2.6 and 4.2 respectively.

Calcium, an alkaline-earth metal, is only bound to the granular biosorbent in very limited quantities (figure 9.12). Although it has the same charge (2+) as most of the other cations investigated in the present study its limited accumulation by the absorbent suggests that the biosorbent is partially selective for cations.

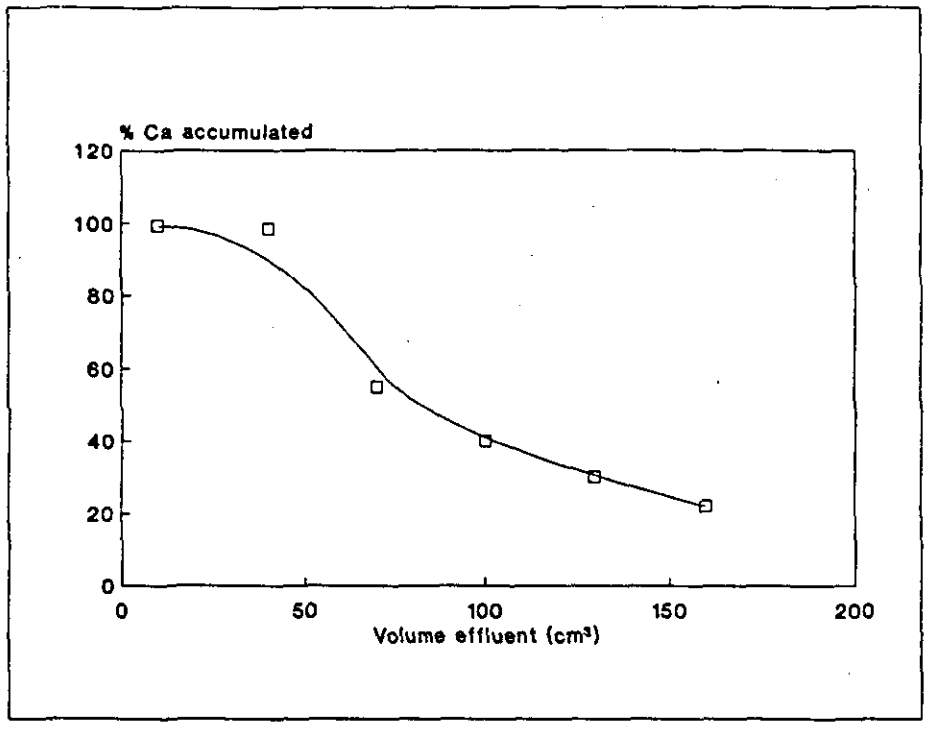


Figure 9.12: Calcium (100 ppm as $CaCl_2$) biosorption by granular biosorbent, influent pH was 7.5.

Channeling appears to occur in granular biosorbent columns. With ferric iron, absorption decreased until the material was physically mixed, after which the level of absoption of iron was restored to > 99% until the capacity of the granular biosorbent was reached (figure 9.13). Similar observations have been made by Brierley *et al* (1986) for MRA who suggested that this effect was due to the small size of the laboratory columns used.

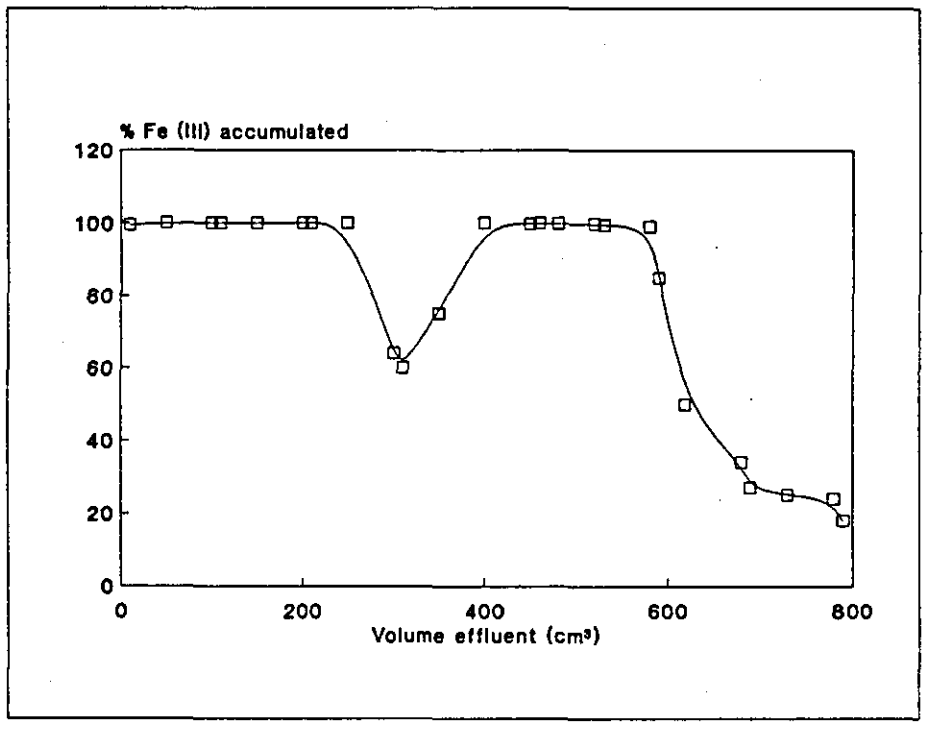


Figure 9.13: Channeling of the influent stream occurs within columns of granular biosorbent, leading to a decrease of biosorption. By mixing the biosorbent into the column, as was done here, ferric iron (100 ppm as ferric chloride) accumulation returned to the > 99% level until breakthrough occurred after biosorbent saturation.

The total cumulative biosorption of metal cations to the granular biosorbent columns of each metal is represented by mole as shown in figure 9.14 and by mass in figure 9.15. From these figures it can be seen that the biosorbent binds certain heavy metals preferentially. Even the same element may be accumulated in different quantities with a change in ionic speciation, the example here being that ferric iron (Fe^{3+}) was bound to a far greater extent than ferrous iron (Fe^{2+}). The alkaline-earth metal (Ca^{2+}) and the only anion tested ($Cr_2O_7^{2-}$) both bound to the biosorbent in minimal quantities.

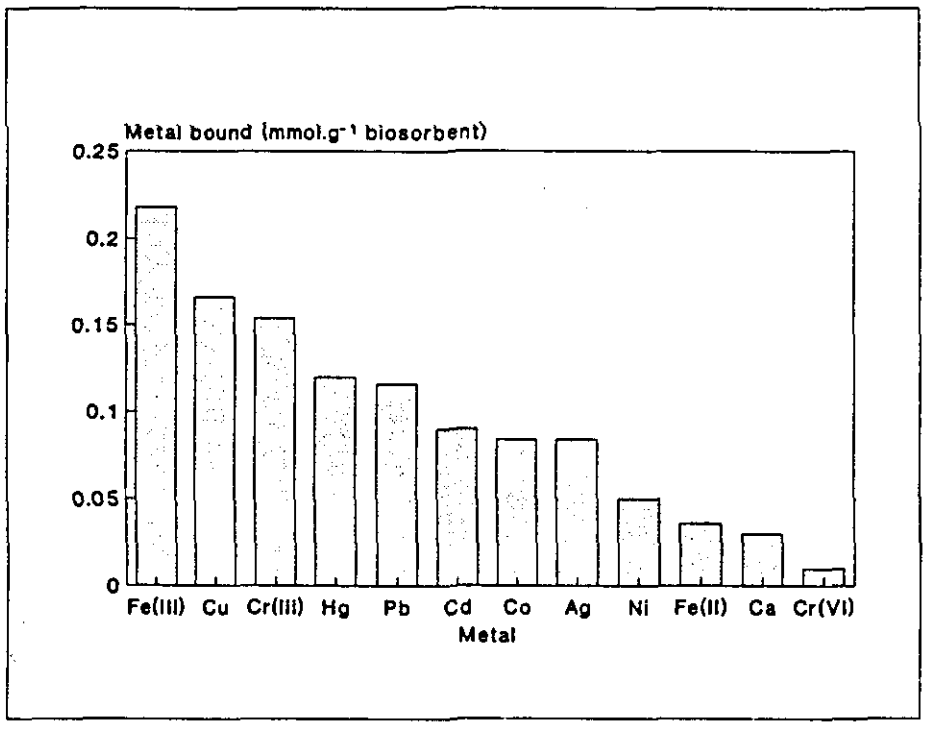


Figure 9.14: Metal ion saturation binding by granular biosorbent as molar quantities of metal bound per gram of biosorbent.

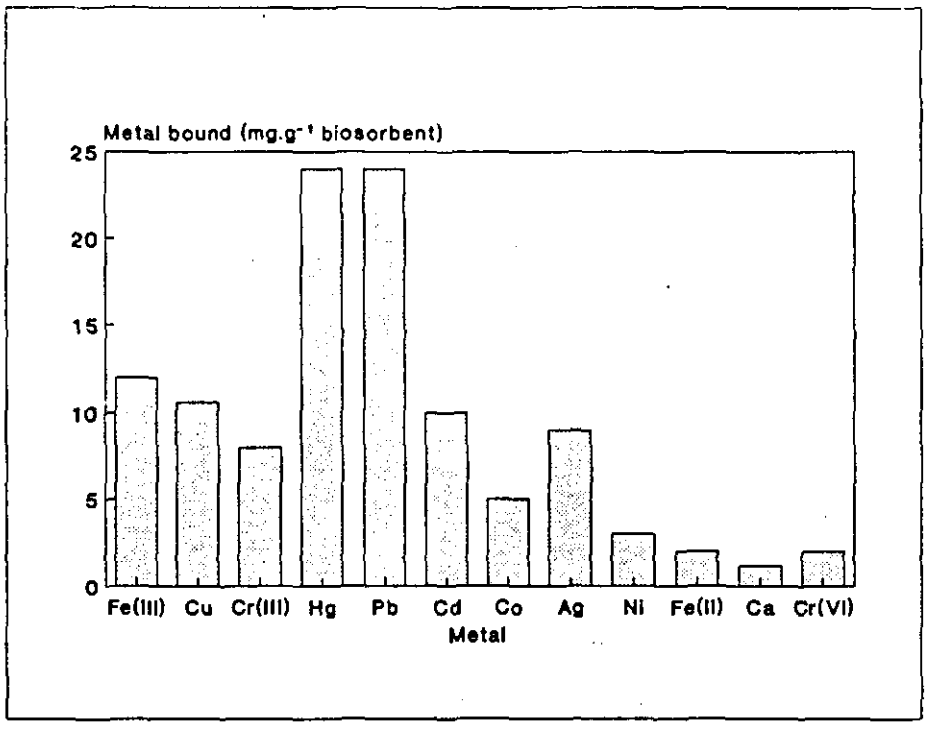


Figure 9.15: Metal ion saturation binding by granular biosorbent as metal mass bound per gram of biosorbent.

The biosorption of individual metals from solutions of mixed metals during batch reactions can be seen in figure 9.16. The binding of all three metals in each group even in the presence of excess residual metal of the other two metals (still in solution) implies that the available binding sites are at least partially specific for each metal. The ratio of binding of the three metals in each case parallels the molar order of accumulation by columns of granular biosorbent (see figure 9.14). The greater levels of biosorption ($mol.g^{-1}$) compared to the figures for columns of biosorbent may represent better penetration of the granules or more complete equilibration owing to the longer contact time in this experiment.

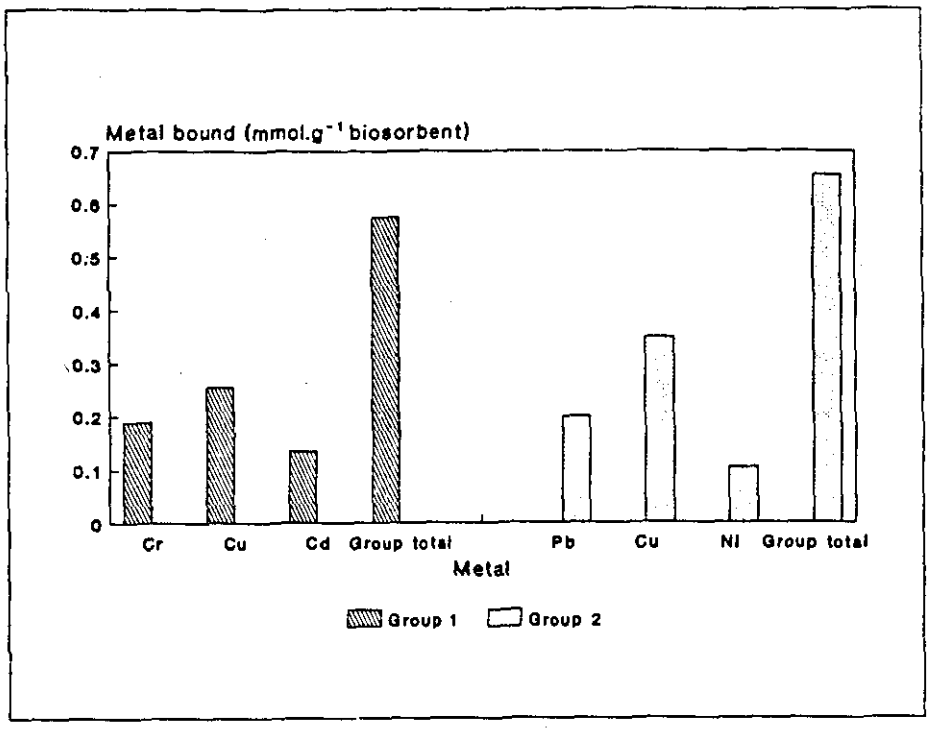


Figure 9.16: Biosorption of metal cations by granular biosorbent from mixed metal cation solutions.

In an experiment on the rate of metal biosorption it was found that metal cations initially bound rapidly to the granular biosorbent but then the rate slowed considerably, possibly due to limiting diffusion rates of the metal into the bulk of the biosorbent (figure 9.17). This suggests that if the porosity of the granules could be increased by modification of the biosorbent preparation method, then the contact time necessary for maximal biosorption could be significantly reduced.

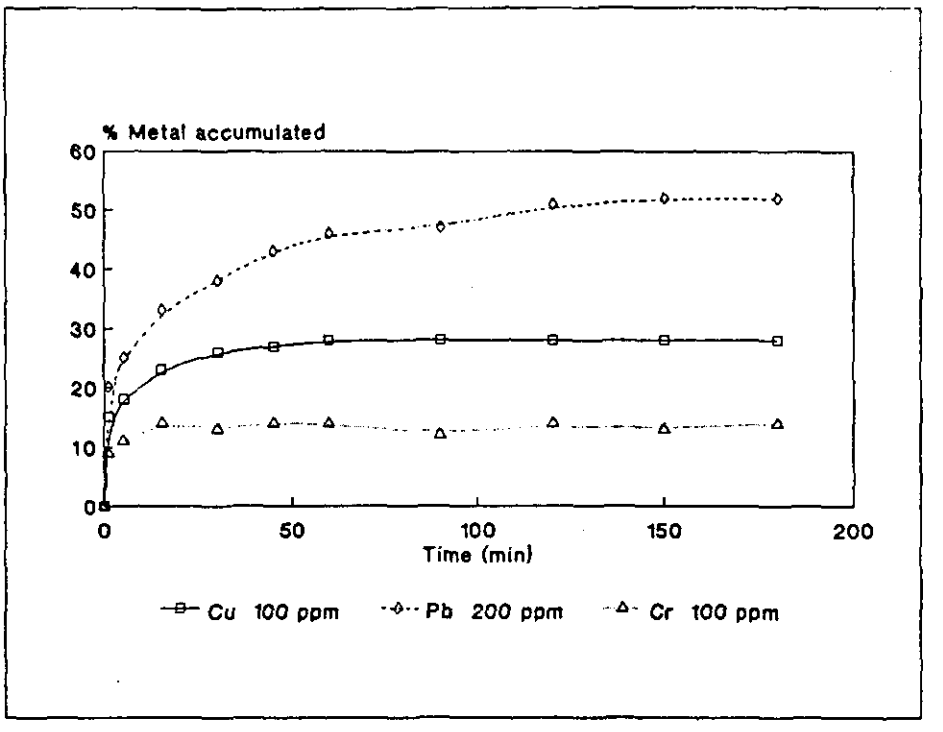


Figure 9.17: Biosorption rate of metal cations to granular biosorbent.

9.4 DISCUSSION

In the present study lower amounts of metal cation accumulation was found using the biosorbent from the yeast *S. cerevisiae* when compared to the results of Brierley *et al* (1986) where a biosorbent from the microorganism *Bacillus subtilis* was used.

As was noted by Brierley *et al* (1986) not all biomass which is converted to granular biosorbents by caustic treatment has the same capacity for metals. However,the order of metal biosorption by mass in this study was similar to that found by Brierley *et al* (1986), i.e.:

Brierley *et al* (1986):

$$Pb^{2+} > Au^{2+} > Cd^{2+} > Cu^{2+} > Zn^{2+} > Ag^{+}$$

Present study:

$$Hg^{2+}, Pb^{2+} >> Fe^{+3}, Cd^{2+}, Cu^{2+}, Ag^{+} > Cr^{3+}, Co^{2+}, Ni^{2+}, Fe^{2+}$$

This suggests a similar mechanism of bioaccumulation by the different biosorbents.

Toxic effects of heavy metals are not a problem with dead biomass or products derived from dead biomass; and such materials may also be resistent to other adverse conditions such as elevated temperature or nutrient limitation (Gadd, 1990b). They therefore have many of the advantages of selective ion-exchangers, but also may have similar limitations.

Similar to the observations of Brierley *et al* (1986) it was noted in the present investigations that during metal accumulation the pH of the effluent increases relative to that of the influent (data not shown), possibly representing ion-exchange with protons. This could be advantageous in the case of acid mine leachate as it would raise the pH towards neutrality with possible consequent precipitation of metal ions as hydroxides.

Caustic-treated biomass has an obvious advantage as a metal binding agent since it yields a pellet that does not have to be immobilized as has been found necessary by other workers using microorganisms killed by other methods (Brauckmann, 1991). However, this method also has

disadvantages. Pre-treatment of *Penicillium* biomass with alkali (NaOH) was found to greatly reduce its metal uptake capacity (Galun *et al*, 1987). A moderate decrease of biosorption capacity for Cu^{2+} after alkali treatment of the biomass was observed in the present study where the capacity of the granular biosorbent was only slightly less than that of the native yeast, but much of the potential binding material released by the alkali treatment is not incorporated into the granules. It would be advantageous to be able to utilize the material lost during alkali treatment, although the development of novel techniques would be necessary to allow for its incorporation into the granular material.

9.5 CONCLUSIONS

The experiments conducted in this study have shown the granular biosorbent to be an efficient remover of heavy metals from otherwise pure aqueous solutions. Calcium without the presence of heavy metal ions showed very little absorption, indicating that adsorption is fairly specific and not due solely to charge.

It is important to determine how this granular biosorbent would perform in accumulating heavy metals from wastewaters and what applications it would be suitable for if it is to be utilized in actual wastewater treatment processes. These questions are investigated in the next chapter.

10. APPLICATIONS OF BIOSORPTION OF HEAVY METAL CATIONS BY NON-VIABLE YEAST

10.1 INTRODUCTION

The application of a biosorbent for heavy metal removal from industrial effluent is the ultimate aim of heavy metal biosorption research. Questions that need to be answered are: what ambient conditions could be expected in the wastewater, and what properties must the biosorbent possess for it to be effective? The aim of this section of the present study was to examine examples of possible industrial usages of the granular biosorbent and investigate the limitations of its use. The metals lead and chromium were investigated in this chapter, the former because it is toxic (see Appendix 2) and is known to contaminate potable water (Nriagu and Pacyna, 1988), and the latter as it is a potential problem for the local tannery industry.

10.2 MATERIALS AND METHODS

The methods used here were similar to those described in chapter 9. Additional details will be described in the results section where relevant.

10.3 RESULTS

In chapter 9 it was shown that the biosorbent derived from *S. cerevisiae* exhibited preference for adsorption of certain cations. What was unclear was whether the cations occupy the same binding sites, partially over-lapping sites, or completely different sites. To answer this question metal-supplemented solutions were passed through columns which were saturated with a different element. Cation competition for binding sites was found to occur. Copper and lead could partially replace each other when passed through granular biosorbent columns saturated with the alternate metal (figures 10.1 and 10.2).

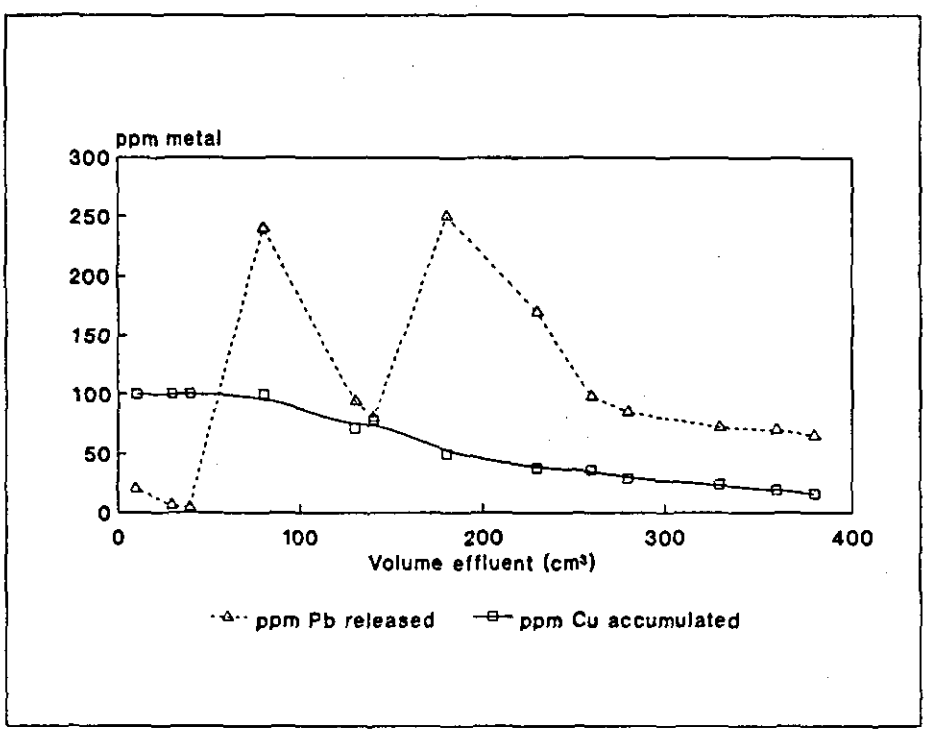


Figure 10.1: Lead release from a lead-saturated column of granular biosorbent during copper (Cu^{2+}) loading.

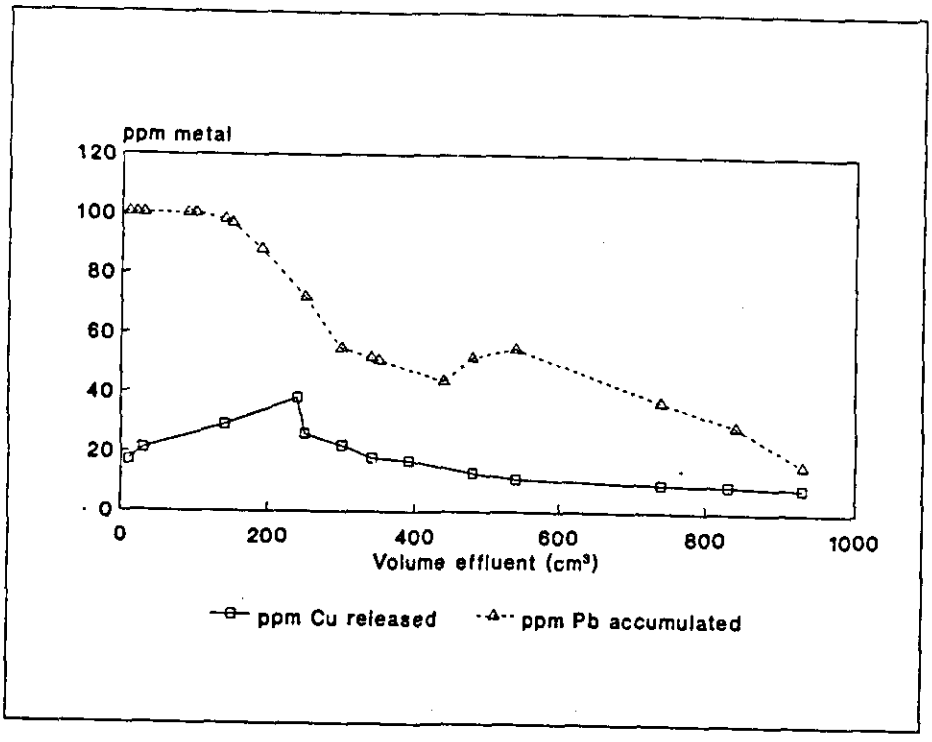


Figure 10.2: Copper release from a copper-saturated column of granular biosorbent during lead (Pb^{2+}) loading.

During Cu^{2+} binding on a lead-saturated column the release of approximately 1 mole of Pb^{2+} for each mole of Cu^{2+} adsorbed means that they may bind at identical or overlapping sites. The uneven release of Pb^{2+} may represent the removal of Pb^{2+} from sites of increasing affinity for Pb^{2+} by influent Cu cations. The blue colour which is characteristic of copper binding to the biosorbent formed evenly instead of the usual progression from the inlet, again implying that there were binding sites to which copper bound preferentially before displacing the lead ions which were bound with greater affinity. The elution of Cu^{2+} from a copper-saturated column of granular biosorbent by Pb^{2+} shows a similar pattern of displacement of one metal by another.

Apart from copper cations, lead could be partially eluted from the granular biosorbent by the chelating agent EDTA or sodium hydroxide (figure 10.3). The most effective lead eluting agent was EDTA, which removed most of the metal from this sample of lead-loaded granular biosorbent.

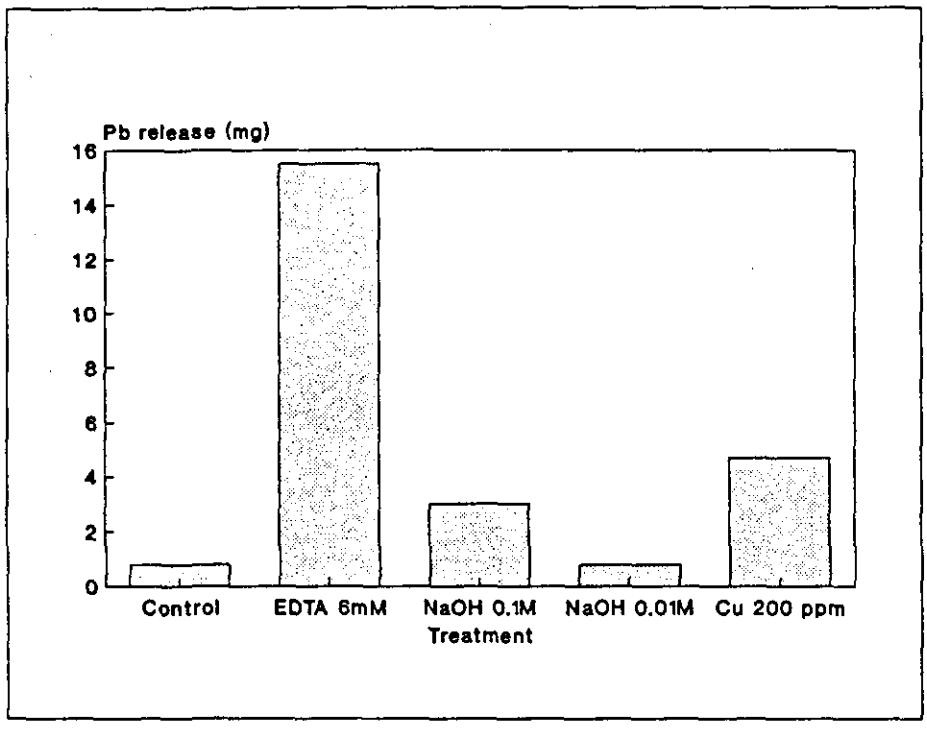


Figure 10.3: Elution of lead from granular biosorbent.

As a practical assessment of the possible uses of the granular biosorbent, its capacity to accumulate lead ions from lead contaminated tap water was investigated. Granular biosorbent could in fact absorb all the lead from the tapwater down to the detection limits of 1 ppm lead or less (figure 10.4).

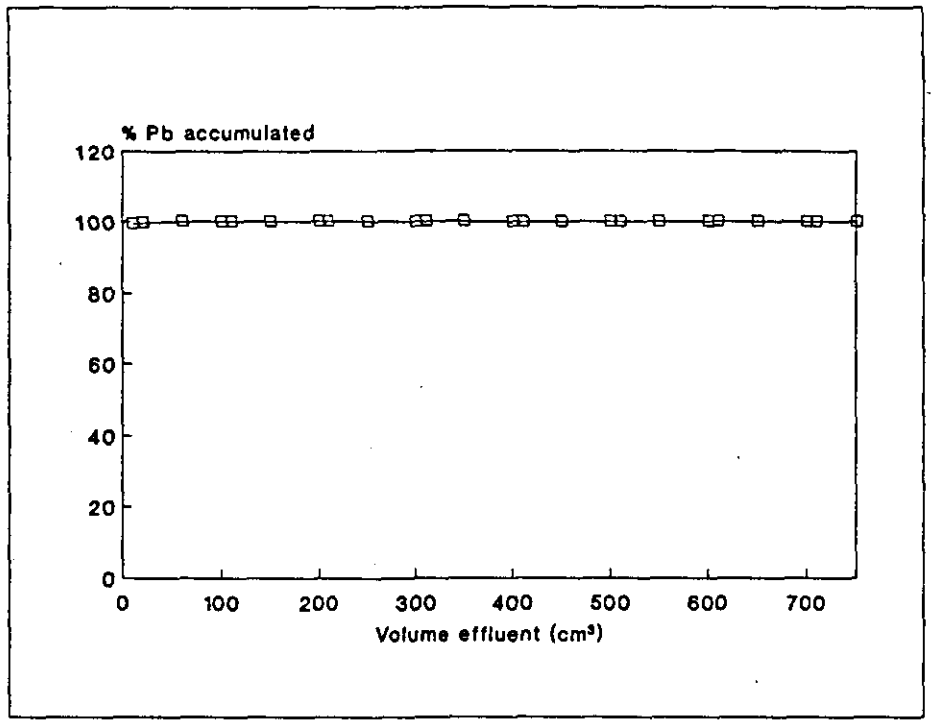


Figure 10.4: Lead accumulation from tapwater by a column of granular biosorbent.

In the range of temperatures which are expected in most common effluents, i.e. between 0 - 40°C, there is little difference in the level of lead biosorption (figure 10.5). The binding of lead to granular biosorbent is, however, to a certain extent endothermic in nature, since binding increased slightly with ambient temperature. Similar increases of binding of copper with increasing temperature were reported by Brierley *et al* (1986).

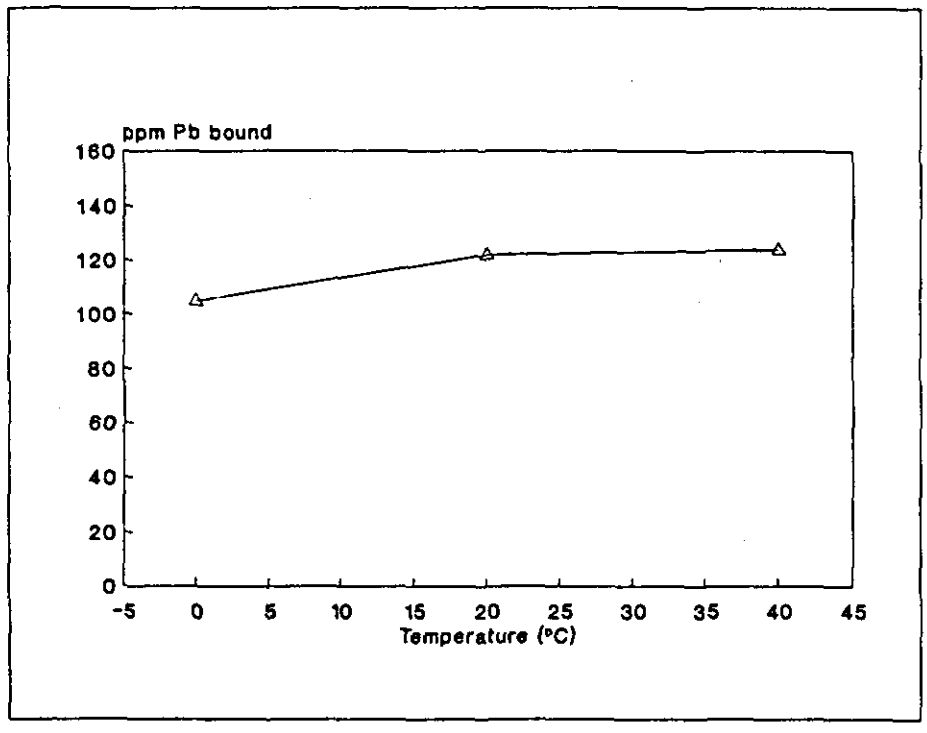


Figure 10.5: Variation of lead accumulation by granular biosorbent with temperature.

Chromium release into wastewaters by the tanning industry is a by-product of the production of "wet blue" leather, a heat stable form of tanning using chromium as the tanning agent. The Cr(III) which is used in the process is released into the wastewater. This in itself in not a significant pollutant, as trivalent chromium is relatively

non-toxic. However Cr(III) can be oxidized in the environment to hexavalent chromate, which is extremely toxic. It is necessary, then, to recover the chromium before it becomes an environmental hazard. The granular biosorbent used in this study was tested as a possible agent for chromium removal from tannery effluent. It was found to be capable of only limited, but fairly consistent, accumulation of the chromium from tannery wastewater (figure 10.6). It appears that about half of the chromium was bound to compounds present in the wastewater that had a greater affinity for the Cr^{3+} than the granular biosorbent. An alternative possibility is that binding sites on the biosorbent are masked by other compounds, such as proteins or tannins, that are found in the effluent. This would interfere with copper binding to the biosorbent.

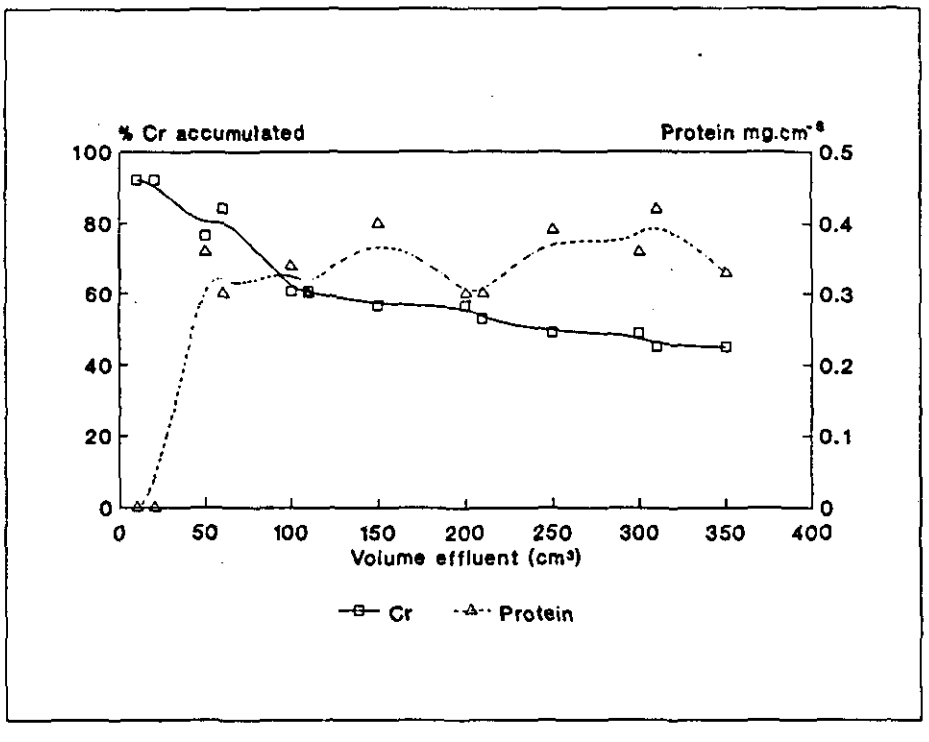


Figure 10.6: Biosorption of chromium from tannery wastewater (post anaerobic digestion) by granular biosorbent. The influent chromium concentration was 2.6 ppm and the influent pH was 7.51.

In the influent tannery wastewater the concentration of Na was 30 $mmol.dm^{-3}$, that of Ca^{2+} was 10.3 $mmol.dm^{-3}$, and that of protein was 0.388 $mg.cm^{-3}$. The interference caused by these constituents of the wastewater on chromium accumulation was investigated. Interference of Cr (III) absorption from a 100 ppm Cr solution by counter ions such as sodium and calcium was minimal at the concentrations tested. Proteins below their isoelectric pH have been found to bind to yeast cell walls (Shaeiwitz *et al*, 1989). However, protein (bovine serum albumin) appeared to have a minimal effect on trivalent chromium binding (figure 10.7) even though bovine serum albumin in itself is capable of chelating metal ions (Verma *et al*, 1982). The possibility that protein may bind to the granular biosorbent and thereby inhibit chromium binding was investigated. The biosorbent, however, did not accumulate protein from either synthetic solutions or tannery effluent (figures 10.6 and 10.8).

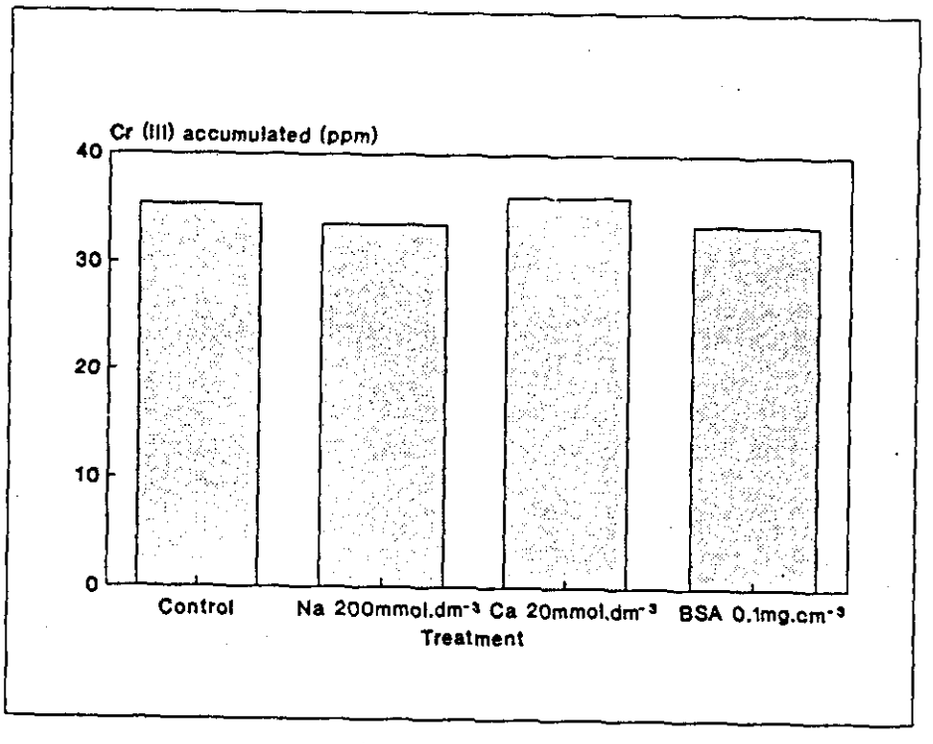


Figure 10.7: *Interference of chromium biosorption by granular biosorbent by certain organic and inorganic compounds.*

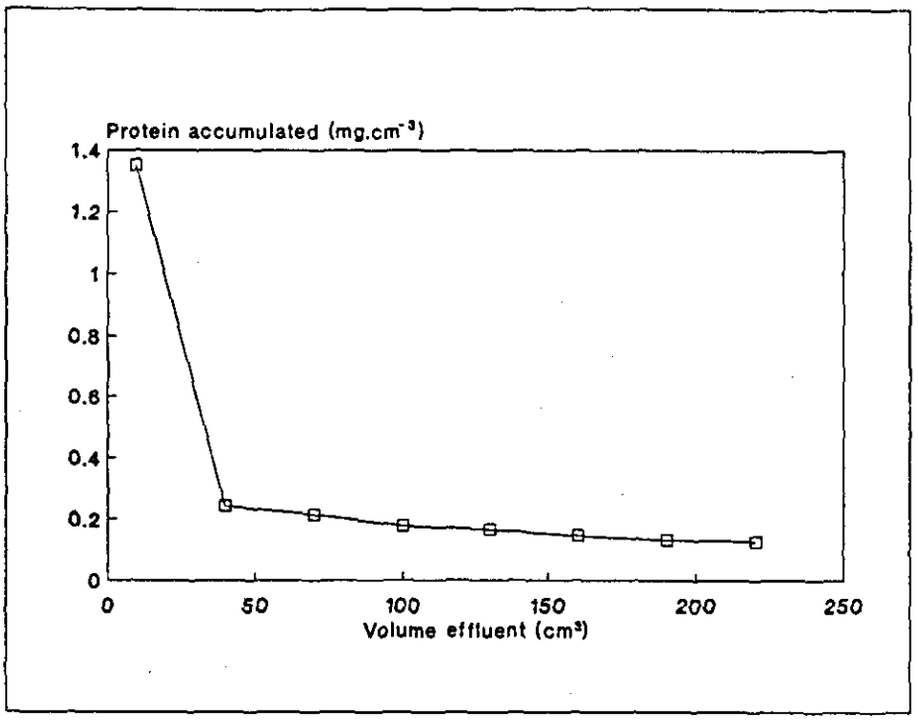


Figure 10.8: Protein binding to a column of granular biosorbent.

10.4 DISCUSSION

The molar ratios of metal released from metal-saturated columns, when compared to the accumulation of the elutant metal cation, reflect the partial specificity of the granular biosorbent and its greater molar capacity for Cu^{2+} than Pb^{2+} as shown in chapter 9. For every mole of Pb^{2+} accumulated 1.04 mole of copper was released from a copper-saturated column, while 1.20 moles of Cu^{2+} accumulated for every mole of lead released from a lead-saturated column. These results also suggest that the assorted metal binding sites for each metal overlap.

Thermal studies have shown that biosorption is an endothermic process (Weppen *et al*, 1990). However, the increase in accumulation of cations with temperature increase is relatively small (Friis and Myers-Keith, 1986). In the present study an increase from 0 - 40°C only increased lead accumulation by 14%, and most of this increase occurred within the first few degrees.

The accumulation of Cr^{3+} from tannery wastewaters by the granular biosorbent was only partially successful as only about 50% of the Cr^{3+} was available for accumulation. Although the possible accumulation-inhibiting effects of some of the constituents found in the tannery wastewater was investigated using synthetic media, none of these constituents exhibited a pronounced effect on Cr^{3+} accumulation. A bacterium commonly found in sludges and anaerobic digestors, *Zoogloea ramigera*, produces an extracellular acidic polysaccharide which can complex heavy metals (Norberg and Rydin, 1984). A similar material may be present in anaerobically digested tannery effluent and may be the reason the granular biosorbent material was unable to accumulate more than 50% of the Cr^{3+} from anaerobically digested tannery wastewater.

The capacity to be recycled is an important aspect of any successful biosorbent. In the present study simple elution with mild acid did not allow for re-accumulation of Cd^{2+} (data not shown). This in part may be due to the fact that glucans, which probably comprise the bulk of this biosorbent, are soluble in acid environments (see section 6.6). Other methods of elution will therefore have to be applied or developed for this biosorbent.

Alternative uses for non-viable yeast based products.

(a) Yeast extract has been used as an important adjunct in microbiological mineral leaching where the active microorganism, *Thiobacillus ferrooxidans*, is detrimentally affected by, and very sensitive to, the trace amounts of silver ions found in certain ores. Addition of small amounts of yeast extract (0.02%) alleviated the toxicity of the silver ion to a large degree, probably by complexion, thus reducing the long lag period of growth to near normal lengths (Tuovinen *et al*, 1985).

(b) Toxic metals can be extremely harmful to the anaerobic digestion process. Anaerobic digestion relies on a careful balance of different microbial populations which may take a long period to reach biochemical equilibrium. The biological compounds in anaerobic digestors may chelate metals and insoluble inorganic salts may form, leading to precipitation. It has been suggested that addition of yeast extract in the growth medium may be valuable for its cation chelating properties, thereby reducing the possibility of heavy metal toxicity to the delicate system (Callander and Barford, 1983).

(c) Although desulphurization of coal by *T. ferrooxidans* has been suggested (Atkins *et al*, 1986; Garcia *et al*, 1989; Andrews *et al*, 1988; Kilbane , 1989) *S. cerevisiae* both as live cells, and more effectively when physically disrupted, has been shown to assist in the desulphurisation of coal fines by decreasing the pyrite (iron sulphide material) content of the coal during froth flotations. This has been found to occur just as rapidly and with comparable efficiency to *T. ferrooxidans* (Townsley and Atkins, 1986).

10.5 CONCLUSIONS

Biosorbents, either in granular form or as soluble flocculators and chelating adjuncts, could fill a large number of roles in metal cation recovery from wastewaters. Unlike selective ion-exchangers, biosorbents do not require expensive and complicated syntheses, but only a simple reaction to cause cell death, followed perhaps by a drying process to reduce volume and mass prior to transport. Yeast biosorbents are also readily available from fermentation based industries.

Biosorbent technology is, however, still embryonic and much improvement could be effected by use of alternative biomass killing methods, novel engineering processes, and a more detailed understanding of the mechanism and factors affecting biosorption processes.

GENERAL CONCLUSIONS

11. BIOACCUMULATION OF METALS BY YEAST: GENERAL CONCLUSIONS

11.1 BIOACCUMULATION

Microorganisms have a variety of mechanisms for mobilizing and immobilizing metals (Woods and Rawlings, 1989; Suzuki, 1986). In this study the ability of the yeast *Saccharomyces cerevisiae* to accumulate heavy metals has been explored.

11.1.1 Free Yeast Cells:

Yeast cells are capable of accumulation of various metals, preferentially accumulating those of potential toxicity and also those of value. They retain their ability to accumulate heavy metals under a wide range of ambient conditions.

In the present study it was shown that yeast cells accumulate heavy metal cations such as Cu^{2+}, Co^{2+}, and Cd^{2+}. The level of copper accumulation was dependent on the ambient metal concentration and was markedly inhibited by extremes of ambient pH. Temperature (5 - 40°C) and the presence of the alkali metal sodium had much smaller effects on the level of copper accumulation. This suggests that in wastewaters with pH 5 - 9, yeast biomass could provide an effective bioaccumlator for recovery of the metal.

However, during bioaccumulation and subsequent processes it is necessary to retain the biomass. This was achieved in the present

study by two alternative methods, i.e. membrane filtration and cell immobilization.

11.1.2 Tangential Flow Microfiltration:

In the present study cross-flow microfiltration was shown to retain yeast biomass after the biomass had been involved in bioaccumulation. The system could also be used to allow for harvesting of the biomass and subsequent bioaccumulation of copper by the biomass on the membrane. The passage of copper-laden influent through a series of sequential bioaccumulation processes allowed for further reduced levels of copper in the final effluent than afforded by a single bioaccumulation process.

Cross-flow microfiltration processes may be of importance in the removal and reclamation of wastewater-borne metal cations, no matter what type of microorganism is utilized for bioaccumulation of metals. The equipment and running costs are relatively low and the system is efficient. Moreover, the equipment could be linked to other systems with relative ease. For instance, yeast or other microorganisms such as algae could be used to accumulate metals from a reservoir and the cells retained by the microfiltration membrane; this would act as the coarse "scrubbing" step. The permeate could then be passed through a fixed bed column of immobilized yeast to completely remove the remaining metal ions. A second microfiltration system would then be of use downstream to retain any heavy metal-laden biomass lost from the column.

11.1.3 Immobilization:

The processes involving membrane filtration used in this investigation, although effective, did not achieve the reduction of cation levels in the aqueous stream that was permitted by column systems involving biomass immobilization. Immobilization allowed for complete removal of Cu^{2+}, Co^{2+}, and Cd^{2+} from synthetic metal solutions. The immobilized material could be freed of metals by use of the chelating agent EDTA and recycled for further bioaccumulation events with little loss of accumulation capacity.

If cost effective methods of immobilization at a scale that will permit the necessary flow rates required by industry can be developed, then this process will be of immense value in heavy metal bioaccumulation. As can be seen in the results presented in this work, this method allows for complete removal of metals from aqueous waste streams owing to its many "theoretical plates" of equilibrium, something which cannot occur with batch bioaccumulation processes.

Although the ability of yeast biomass to accumulate metals at very low ambient concentrations was not investigated in this study *per se*, the fact that columns of immobilized yeast cells can effectively eliminate heavy metals from aqueous solutions indicates that they do accumulate heavy metals from low metal concentration solutions, and it is only the overall efficiency of the biomass to accumulate heavy metals from these low ambient concentrations that is in question.

Comparison of experimental results with those from the literature reveals that different microorganisms appear to accumulate metal

cations with different specificities. It may thus be possible to use a series of fixed bed columns of immobilized cell types to specifically accumulate required metals.

11.1.4 Heavy Metal Tolerance :

The development of metal-tolerant strains for bioaccumulation is problematic. In the present study the aquisition of tolerance to Cu^{2+} involved decreased Cu^{2+} uptake by the yeast, a response which is obviously undesirable for effective bioaccumulation. One mechanism of heavy metal toxicity resistance is the induction of chelating agents. In yeasts, however, the induction process appears to be metal-specific so that resistance to one heavy metal will not result in synthesis of the same chelator if the yeast is challenged by another type of metal. In summation, it is not profitable to develop tolerant strains of microorganisms unless they are to be used in continuous cultures where the influent wastewater has a constant heavy metal composition.

11.2) THE MECHANISM OF ACCUMULATION OF HEAVY METALS BY YEASTS

To maintain and optimise any industrial process it is necessary to have a thorough understanding of the mechanisms and principles that govern the process. In the present study experiments were performed to add to the body of knowledge on these mechanisms. The process was examined in *S. cerevisiae* by studying the metal binding of various fractions of yeast cells in a sequence likely to parallel that encountered by the influent metal.

11.2.1 Cell Wall and Cell Wall Components:

The cell wall is an important component in metal cation binding. It is with this cellular fraction that influent metals first come into contact with and bind to. It also appears that the cell wall is both capable of and responsible for, binding of metal cations at lower ambient levels than are bound by the cell interior.

Based on the results of this study it appears that most of the cell wall components are responsible for some copper accumulation. While nitrogen groups were found to be effective in copper accumulation, hydroxyl and carboxyl groups were also important (Brady and Duncan, 1991) and may be relatively more important for other metals than the copper which was investigated in this study. It also appears that the physical state of the cell wall may be just as significant as the chemical ligands for heavy metal binding.

Modification of the cell wall was found to modify the affinity of the cell wall for metals. However, it is unlikely that such modifications would be economically viable.

11.2.2 Sequestering Proteins:

Once the metal enters the cytosol it may be bound by intracellular chelators as was found in this study. The usually low levels of these proteins in the cell would mitigate against their importance in industrial bioaccumulation of metals from wastewaters. If the intracellular quantities of such proteins could be greatly increased by such methods as genetic engineering, then they may be of some use.

Here again, however, such modification would possibly be prohibitively expensive. If the chelators were of sufficient specificity they could be of profound use as detoxifiers in medicine or as part of sophisticated biosensors.

11.2.3 The Vacuole:

As the "ion storehouse" of the cell the vacuole appears to be potentially capable of accumulating vast quantities of metals. A great deal more work is necessary to determine the exact role of these intracellular organelles in the bioaccumulation of metals. Clarification is required in terms of which metal species the vacuole assimilates, and under what physiological conditions.

Although in the present study no investigations were performed on isolated vacuoles, the release of certain cations which are specifically compartmentalized into the vacuole or cytosol allowed tentative conclusions to be made about the role that these two compartments may play in uptake of heavy metals such as copper. Upon contact with copper-containing solutions the yeast biomass rapidly lost intracellular K^+, which is mainly compartmentalized in the cytoplasm, suggesting that the plasma membrane is affected by the Cu^{2+} ions. Then there is a much slower release of Mg^{2+}, which is stored in the vacuole, which may therefore represent a slow exchange of ions across the vacuolar membrane. Studies on metal bioaccumulation by isolated vacuoles and the use of autoradiographic techniques on whole cells may allow for a better understanding of the process of heavy metal uptake and eventual compartmentalization of the accumulated metal.

11.3 BIOSORBENTS

Non-viable cells have many obvious advantages over viable cells, such as their lack of autolytic enzymes and their invulnerability to metabolic toxins present in industrial and domestic wastewaters. They may also be stored indefinitely. Killed cells and their by-products can be used in many different ways and forms of product. They can be converted into pellets, as in the present study, into films, or used as heavy metal flocculents.

Results from this and other studies have shown that such biosorbent material can be used as an effective metal-accumulating agent. The disadvantages of these biosorbents are that they must be chemically treated which increases their cost on a mass basis, and that some of the accumulation mechanisms which are functional in metabolically active yeast are lost. Biomass drying processes may,however,help to cut costs if transport of the material is required.

11.4 GENERAL COMMENTS

Conventional methods for removal of heavy metals from wastewater streams include chemical precipitation, chemical oxidation or reduction, ionic exchange, filtration, electrochemical treatment, and evaporative recovery. Such processes may be ineffectual or extremely expensive when initial heavy-metal concentrations are in the range of 10 - 100 (ppm) and discharge concentrations are required to be less than one (ppm) (Shumate *et al*, 1978). This area of operation is

therefore open to competition from bioaccumulation processes.

An advantage of bioaccumulation processes is their specificity for heavy metals. However, specificity of metal accumulation by organisms is relative. Although most microorganisms (including the yeast in this study) preferentially accumulate heavy metals, cation competition will always occur if the competing cation is in sufficiently high concentrations. The bioaccumualtion of metals from wastewaters would therefore be most effective if the biomass was in contact with relatively pure and concentrated metal ion solutions. This demands separate treatment of individual wastewaters as far upstream in the process as possible. In effect metal bioaccumulation processes would be most cost effective and competitive at metal concentrations just below those at which traditional processes are economical.

It many respects the desorption of metals from biomass may be as important as the bioaccumulation process. The use of certain elutant solutions allows for specific desorption of specific metals from biomass, thereby increasing the specificity of the bioaccumulation system considerably.

Chlorella vulgaris has been shown to be capable of accumulating a wide variety of heavy metal cations. Darnall *et al* (1986) have found that if the alga has simultaneously accumulated a variety of metal cations, that the metals may be selectively desorbed. Most metals, such as Cu^{2+}, were eluted by reducing the pH to 2, while others, such as Hg^{2+}, Au^{2+} and Ag^{+} remained firmly bound. The gold and mercury could then be selectively eluted by addition of mercaptoethanol (which presumably dissociates the metals which bind more firmly to thiol groups on the

biomass). This added selectivity may make the biomass more viable than synthetic ion exchange resins which usually possess a single ligand type.

During desorption of metals it is imperative that minimal damage occurs to the bioaccumulation properties of the biomass if the biomass is to be reused in further cycles of metal adsorption and desorption (Tsezos, 1984). This requires that the metal from the biomass be eluted under mild conditions. As a direct consequence of this, the concentration factor for biomass is much lower than for ion-exchange resins, which are eluted by harsh chemical treatments such as concentrated acid. The relative structural weakness of biomass also means that while ion-exchange resins can withstand hundreds of adsorption-desorption cycles without significant damage, biomass may lose a sizeable percentage of its dry mass within a few cycles. The capacity (by mass) of biomass for metals can be equal or superior to that of selective ion-exchangers, but it has the disadvantage that its volume to mass ratio is comparatively much greater (Röhricht *et al*, 1990). This in turn would necessitate larger contact vessels with concomitant cost increases. However, waste yeast can be much cheaper than metal selective resins (Röhricht *et al*, 1990).

Investigations into the optimal elutants and their concentrations should be a high priority in future bioaccumulation research. Determination of the effectiveness of various elutants would not only be of practical value, but may also help to further elucidate the mechanisms of heavy metal adsorption.

The bioaccumulation of the rare earth metals has been almost totally ignored, but recently Karavaiko *et al* (1991) have shown that a wide range of fungi and yeasts accumulate yttrium and scandium from solutions with high affinity; accumulation was not affected by the presence of Fe^{3+}, but Ti^{2+} reduced accumulation.

In general it can be concluded that while yeast has been used by mankind for over six thousand years for a variety of biochemical processes, and is presently one of the most important commercial microorganisms, its importance and industrial utilization could further increase. This is because yeast is not only an ideal organism for producing certain compounds, which can only be synthesized by eukaryotes (Grivell and Planta, 1990), but there is the additional possibility of the application of this organism to biohydrometallurgy.

It should be borne in mind that biological bioaccumulation processes need not be complete systems on their own, and that a particular process may not be applicable to all situations.

Widespread commercial acceptance of biological metal accumulation systems has not occurred. The reasons for this are the lower metal uptake capacity and suspected fragility of the organisms, but these problems could possibly be overcome if there was improved knowledge of accumulation mechanisms and the parameters for process scale up. The use of *T. ferrooxidans* in ore leaching took many years and a few failed attempts before economic success was finally achieved (Lakshmanan, 1986). The industrial community will only accept bioaccumulation systems that are reliable and technically sound.

This study has attempted, at least in part, to more clearly define the heavy metal bioaccumulation potential of yeast and yeast products, and to elucidate the mechanisms of this accumulation, with a view to the eventual commercial utilization of this biomass in metal removal and/or recovery from wastewaters.

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