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

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 (Odendaal, 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/mining processes produce heavy metal containing wastewaters which represent a highly toxic effluent on the one hand and in the case of the mining industry, a loss of valuable metals. More stringent control of effluent quality and the increasing demand in South Africa for high quality potable water makes the removal of toxic metals from wastewater a major priority.

Methods of metal removal such as ion exchange and precipitation have in many cases either not been efficient or cost effective. Biotechnology based processes however, appear to offer an economic and efficient alternative to these methods. A number of micro-organisms have been shown to actively accumulate ~~mel~~ metal ions and to efficiently remove these ions from solutions and effluents thereby decontaminating the effluent with regard to its heavy metal composition (Hutchins *et al*, 1986; Nakajima and Sakaguchi, 1986). Effective systems have also been devised for using such biotechnological approaches on a continuous basis and for recovery of the metal from the biomass.

Yeast has been chosen as the organism of choice in this study since it is a waste product of the baking and alcohol fermentation industry making it a readily available and inexpensive source of biomass for such studies. Moreover, yeasts have been shown to be very effective and selective in their ability to accumulate certain heavy metals (Norris and Kelly, 1977;

Norris and Kelly, 1979; Nakajima and Sakaguchi, 1986; Jones and Gadd, 1990; Rothstein and Hayes, 1956; Gadd and White, 1989). They are also able to accumulate metal ions from both concentrated and dilute solutions. Of particular value appears to be the possibility of using modified, dead yeast cells or cell components for metal accumulation, a system which has no requirement for costly supplementation of the wastewater with growth nutrients. Previous studies on yeast biohydrometallurgy have not examined this later possibility nor have they revealed the full potential and range of yeast metal bioaccumulation.

It is therefore intended, through these studies, to develop a system for the practical utilisation of a yeast biomass or cell component for the removal of metal ions from wastewater. Such a system should be able to be used on a continuous or semi-continuous basis and the biomass should be reusable. This would provide a means of detoxifying heavy metal containing effluents or could be used as a final polisher in water purification systems. Bearing in mind the inexpensive biomass to be used, it is likely that this would result in a significant reduction in the cost of ion-exchangers or metal precipitating chemicals required by more traditional processes. It may also provide a means of metal removal from, for example, effluents with low metal ion concentrations which may not be possible by conventional means. The possibility also exists for desorption of the metals from the biomass and subsequent recovery of high value metals.

OBJECTIVES

(1) To identify cellular components and chemical groups responsible for metal accumulation by yeasts and to determine the biochemical mechanisms responsible for metal binding/uptake.

(2) To determine the efficiency and kinetics of heavy metal accumulation at varying metal ion concentrations and to compare the mechanisms of accumulation at the different concentrations.

(3) Based on results of the above studies to determine the most efficient system of metal removal from solutions using a column of immobilized cells/cell components. The effective desorption of the particular metal from the column could then be investigated with a view to obtaining a final, highly concentrated solution of the metal and in order to use this system on a continuous basis.

(4) Concurrently with (2) and (3) would be to begin using a yeast biomass system for removal of metals from industrial wastewaters. The wastewaters to be investigated to be identified, in consultation with industrial and Commission personnel, as those of prime importance for detoxification or metal reclaimation in the South African context. The development of pilot plants from the findings of such studies would be a longer term aim.

RESULTS AND CONCLUSIONS

Previous studies in our laboratory have shown that live yeast cells and a modified non-viable form of yeast are able to accumulate a wide range of metal ions.

Further investigations investigated the role of cell walls and cell wall components of yeast in metal accumulation. 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 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.

Experiments on the extraction of certain cell wall components for binding experiments or removal of some components by enzymes before reacting to the cell wall with metals suggests that all the major types of macromolecules found in the cell wall are important in metal binding. Isolation of the three main cell wall components (chitin, glucan, mannan) was

achieved using acid and alkali solubilization and fractionation procedures. Metal uptake was found to be greater by the isolated components compared to the whole cell walls, and copper ions were bound more than cobalt or cadmium ions. It does appear however, that the integrity of the cell wall is critical for effective metal binding.

Studies using X-ray emission electron imcroscopy and isolated vacuoles have indicated that uptake of cations from solution into the cell is effected by a H^+/antiport system located within the cell membrane which is affected by the proton (H^+) gradient. Evidence of the latter system was supplied by monitoring the pH during bioaccumulation. The longer the yeast cells were exposed to the effluent the higher the extracellular pH, indicating an H^+ efflux from within the cells.

On exposure to concentrated Cd^{2+} and Cu^{2+} solutions, loss of intracellular ions from within the cell was observed. The rapid loss of K^+, Na^+ and Mg^{2+} from the cytosol was similar to the trends found by a number of others (Norris and Kelly, 1979, Okorokov, 1985) and which can occur via the ion exchange channels. Although heavy metals increase the permeability of the cellular membranes, e.g. Cd^{2+} which binds to organic ligands (Gadd and Mowll, 1983, Joho, *et al*, 1985, Ohsumi, *et al*, 1988) this did not appear to be the route along which intracellular efflux occurred. Exposure to Co^{2+}, a toxic divalent heavy metal cation, did not result in the efflux of intracellular cations. If this cation had mirrored the behaviour of similar cations by increasing membrane permeability, numerous intracellular ions would have leaked from the cell. Instead it appeared as though Co^{2+} had an inhibitory effect on the ATPase H^+/antiport system. Entry of Co^{2+} into the cytosol appeared to occur via the antiport system, after which the Co^{2+} blocked the channel preventing the release of any intracellular ions.

The effect of glucose pretreatment substantiated the theory that the majority of heavy metal cations enter the yeast cell via the antiport system and not through membrane lesions. Cells pretreated with glucose, an energy source, exhibited a superior ability to remove metal ions from solution. This increased ability of energized cells to accumulate additional metal cations is an indication of metabolic accumulation.

Uptake of heavy metal cations from solution by isolated vacuoles presented evidence of a mechanism different from that of the cell wall binding and intracellular accumulation. Whilst inhibiting vacuolar Cu^{2+} uptake, DNP, an ATPase uncoupling agent, failed to inhibit the uptake of Co^{2+} and Cd^{2+} into the vacuole. This suggests that the latter gained entry into the vacuole via an alternative pathway(s). Cu^{2+}, although affecting the permeability of the plasma membrane, does not affect the permeability of the tonoplast. On addition of DNP, entry of Cu^{2+} into the vacuole was therefore limited. Entry of Co^{2+} and Cd^{2+} into the vacuole was probably facilitated through lesions in the tonoplast. Deposition of metal cations within vacuoles occurred over time. Due to the vacuole occupying a large portion of the cell, a high amount of metal cations were sequestered within this organelle. Once within the vacuole, the positively charged ions associated with the negative polyphosphate bodies, which could explain the large phosphorous peak present in the EDAX spectra of all cells.

Thus it can be concluded that the cell wall is the first cellular structure to come into contact with the metals present in the extracellular solution. All cations appear to bind to the wall in a similar fashion and only differ in the amounts bound. Entry into the cell is facilitated by a H^+/antiport system. Subsequent efflux of intracellular physiological cations (Na, K, Mg) occurs in response to the accumulation of some species of heavy metal cations.

Once inside the cytosol, the cations are taken up by the vacuole either along a H^+ gradient, which is created by a V-ATPase H^+/antiport system, or alternatively through permealization of the tonoplast. The heavy metals are sequestered within the vacuole to reduce cellular toxicity.

Further studies have shown that 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 accumulator. 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 ($\pm$ 300 ml of 100 mg/ℓ solution in a 15 ml column). 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

Results show that flux rates remain constant and that metal bound yeasts do not form an impenetrable flocculate layer. Initial filtration characteristics are maintained. Yeast cells loaded to a cross-flow hollow fibre membrane system present a useful method for removing and recovering certain heavy metals from water. The process offers a system that could feasibly be engineered on a large scale.

Application of the yeast biomass to heavy metal removal from industrial effluents revealed that the yeast was able to accumulate zinc and lead from a zinc refinery effluent as well as a number of metal ions from various electroplating industry wastewaters. Removal efficiencies of 50 - 70% were obtained. While this is somewhat lower than that obtained using solutions containing single metals, it is not unexpected since high concentrations of a number of metal ions found in these effluents affect the uptake of the metal of interest by competing for common binding sites.

Using an immobilized column of non-viable yeast biomass a removal of 50 - 65% of the chromium in a tannery effluent was obtained. It appeared that there was some interference in the chromium binding by the high levels of sodium and the proteins and tannins present in tannery effluent. In terms of the utilization of non-viable cell biosorbents for wastewater treatment, they 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.

In conclusion, this study has shown that all of the cell wall components of yeast cells can bind metal ions with the outer protein-mannan layer being the most effective chelator. Metal cations may also enter the cell by a H^+/antiport system and once inside the cell they can be taken up by the vacuoles. The yeast cells can be immobilized and in such a system it is possible to use them on a semi-continuous basis. Batch and immobilized biomass has been successfully used to accumulate metal ions from industrial effluents although these systems need to be further refined.

recycled for further bioaccumulation processes with minor loss of bioaccumulation capacity.

Cross-flow microfiltration units were successfully utilized to remove metal ions from solution. Yeast cells were blended into a stream of heavy metal-laden water in a reaction vessel with a HRT of 10 minutes. Previous determinations of metal binding isotherms had shown a 55% removal of copper. The yeast-metal suspension was then passed to the filter (flux rate: 9.1×10^2 $1.m^{-2}.hr^{-1}$). Filtration was achieved with limited damage to the yeast and no passage of particulates (measured spectrophotometrically at 540 nm). In the use of copper a removal efficiency of 65% was determined with cation binding of 20 g copper per kg dry mass of yeast.

Using a packed system yeast cells were loaded onto the hollow fibre membrane filter at 0.42 $kg.m^{-2}$ yeast dry mass/filter surface area and flux rates stabilized at 12.3×10^2 $1.m^{-2}.hr^{-1}$ before addition of heavy metal to the water.

The following removal efficiencies were recorded :

Metal	% Recovery	g metal recovered/kg yeast dry mass
Cu	75%	27.2
Cr	71%	21.1
Pb	63%	74.6
Cd	7%	4.5

These levels were sustained at flow volumes of over 3095 $1.kg^{-1}$ dry wt cells without breakthrough at the above metal/yeast mass ratio. Control experiments with metal solutions in 5 mM PIPES buffer (pH 6.5) showed substantially similar results.

Measurement of Ca^{2+} and K^+ showed early release peaks possibly indicating both ion exchange and cellular uptake functions operating in the removal process.

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This study has consequently achieved the initial objectives and has answered many of the questions raised therein. It has further identified the metal binding components of yeast biomass and has elucidated the mechanism of metal uptake and accumulation by these cells. Successful immobilization of the biomass has been achieved and desorption potential has been demonstrated. A potential for use of this biomass for industrial effluent treatment has been identified.

RECOMMENDATIONS FOR FURTHER RESEARCH

(1) Different types and forms of microbial biomass should be investigated for their potential to remove heavy metals from a variety of industrial wastewaters.

(2) The mechanisms of bioaccumulation of heavy metals by different types of biomass should be identified in order to determine the most effective systems to use for the treatment of particular heavy metal containing wastewaters.

(3) Cross-flow hollow fibre membranes and other systems of biomass immobilization for purposes of large scale and continuous use should be more fully evaluated.

(4) Methods for heavy metal desorption need to be investigated to facilitate means of concentrating the metal and also for possible reuse of the biomass.

(5) A pilot scale system for effluent treatment needs to be developed.

(6) The technology developed through these studies should be transferred to relevant industries.

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CHAPTER 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. The importance of this problem is highlighted by the fact that humans are now the largest agent in the biogeochemical cycles of trace metals on 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. 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 high 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

Traditional methods of metal removal from solutions, such as ion exchange and precipitation, have not proved cost effective especially in the lower concentraiton ranges. Biotechnology based processes can however play a role in metal recovery.

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.

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).

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.

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) micro-organisms 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 an organism such as *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).

1.4 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).

The results of studies done in our laboratory have to date indicated that yeast cells and a modified, non-viable yeast cell mass provide an efficient means of removing a wide range of metal ions from solution (Brady and Duncan, 1993a, b). The modified, non-viable biomass had a slightly reduced capacity for metal accumulation but appeared to be a useful source of biomass for biosorption purposes as it can be readily used on a large scale and is easily stored (Brady and Duncan, 1994b). Temperature was found to have a minimal effect on metal accumulation while binding was most efficient in the pH range 5-9. Induction of metal tolerance in the yeast cells resulted in a reduced capacity for metal binding.

The yeast cell wall was found to be the most important component for metal accumulation, with internalisation of the metals only occurring at high ambient concentrations (Brady and Duncan, 1994a). Internalisation occurred through binding to intracellular proteins and uptake into vacuoles. Modification and blocking of cell wall active groups by chemical agents revealed that the amino groups of chitosan and proteins, the carboxyl group of proteins and the phosphate group of phosphomannans were the most important metal binding groups. Enzymatic degradation of the individual cell wall components demonstrated that the cell wall was required to be intact for maximum metal binding capacity.

Extraction of cell wall binding components was successfully achieved and all of the major components were found to be capable of metal accumulation. However, the intact cell wall had a higher binding efficiency than any of the individual components.

1.5 RESEARCH AIMS

The principle aims of the studies described in this report were to confirm and extend the earlier finding in our laboratory on the mechanisms of bioaccumulation of heavy metal cations by *S. cerevisiae* as well as to examine potential immobilisation systems for retention of biomass for more practical application purposes. A further aim was to conduct a preliminary investigation of the potential of this source of biomass to remove metal ions from selected effluents.

The yeast *Saccharomyces cerevisiae* was 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 analytical results and uniformity of permeation in fixed-bed biomass columns.

CHAPTER 2

BIOACCUMULATION OF METAL IONS BY YEAST CELL WALLS AND CELL WALL COMPONENTS

2.1 INTRODUCTION

S. cerevisiae, like many other microorganisms, accumulates various heavy metal ions via two distinct processes. The first is a rapid accumulation step which involves binding of the metal to cell surfaces, and extracellular matrices (i.e. biosorption). This initial process is both temperature and metabolism independant and is followed by slower intracellular uptake, via active or passive processes, which are metabolism dependant (Gadd and Griffiths, 1978, Norris and Kelly, 1979, White and Gadd, 1986). In terms of the practical application of bioaccumulation systems it is the initial rapid accumulation to the cell wall (biosorption) which is of primary significance.

Isolated cell walls of *S. cerevisiae* are capable of binding a wide range of heavy metal ions (Rothstein and Hayes, 1956). The overall nett negatively charged cell wall enables cation exchange and co-ordination of metal ions with negatively charged groups. Binding of these cations to the cell wall is both rapid and reversible (Blundell and Jenkins, 1979). However, the knowledge regarding the binding patterns of these cations to the wall and its individual components is rudimentary.

The yeast cell wall which is approximately 70 nm thick (Phaff, 1971), has been described as one of the most tough and rigid of all microbial cell walls (Hunter and Asjengo, 1988). The cell wall of *S. cerevisiae* consists of a number of polymers including glucan, mannan, protein, lipids and chitin/chitosan (Table 2.1) (Northcote and Horne, 1952, Korn and Northcote, 1960). These components are arranged in bilayered structures surrounding the cell (Hunter and Asjengo, 1988). Mannans which are covalently linked to peptide units forming mannoproteins and the glucans are the predominant structural components within the wall.

Table 2.1: The chemical composition of the cell wall of *Saccharomyces cerevisiae* (Northcote and Horne, 1956)

Component	% Dry Weight of Cell Walls
Nitrogen	2.1
Phosphate	0.31
Protein	13.0
Ash	3.21
Lipid	8.5
Mannan	31.0
Glucan	29.0
Chitin	1.0
Chitosan	1.0

The alkali insoluble glucans, consisting of polymers of β(1-3) linked glucose with β(1-6) branches are found primarily on the cell membrane side of the rigid bilayered cell wall. Besides being the component responsible for maintaining the shape and rigidity of the cell, the layer of glucan microfibrils prevents the cell from undergoing osmotic lysis and mechanical breakdown (Hunter and Asjengo, 1988).

Both alkali-soluble and alkali-insoluble forms of glucan exist in the wall, the β(1-6) linkages being responsible for the alkali-insolubility of the glucan (Bacon *et al*, 1969). Cately (1988) identified two further types of glucan in yeast cell walls, namely α(1-3)- and α(1-6)-D-glucans. Microscopic observation has shown that the glucan component is aggregated into microfibrils and is located toward the inner surface of the cell wall (Hunter and Asenjo, 1988).

Mannan (a mannose polymer) is found in association with protein in yeast cell walls. The mannan-protein complex is located on the outer surface of the wall and appears to cover the entire surface. The outer mannoprotein layer consists of a core of 15 - 17 β(1-6) mannose units linked to the asparagine moiety of the peptide through the di-*N*-acetylchitobiose unit. The mannan:protein ratio of this complex is 12:1, thereby forming a molecule with a slightly

larger dimension than pure manna (Phaff, 1971, Cabib and Roberts, 1982).

A small amount of phosphate (0.2 - 1.0%) is found in the mannoprotein complex (Northcote and Horne, 1952), but not in the form of phosphoprotein as initially thought. Instead the phosphodiester bonds are responsible for cross-linking some of the mannan side chains (Phaff, 1971, Cabib and Roberts, 1982).

The third sugar polymer, chitin consisting of long chain unbranched β(1-4)-*N*-acetyl-glucosamine residues, is only found to exist in small amounts (1 - 2%) in most yeast cell walls. These polymers are highly insoluble, either as chitin or chitosan (the deacetylated form of chitin) and are predominantly found in bud scars conferring resistance against chemical attack upon them (Northcote and Horne, 1952, Bartnicki-Garcia, 1962, Phaff, 1962, 1971, Cabib and Roberts, 1982). Chitin is thought to be associated with the glucan component of the cell wall due to the unavoidable degradation of glucan observed during chitin extraction (Bacon *et al.*, 1969).

For bioaccumulation to become economically viable, it would be required to show several advantages over existing technologies for the removal of metals from wastewaters (e.g. precipitation, adsorption, ion exchange and membrane technologies) would have to be exhibited (Rohricht, 1990). Two essential properties of yeast would thus have to be a high initial uptake of metal by cell wall components, and the easy removal of metal ions from the components allowing for their reuse within the system (Gadd, 1989).

Previous studies in our laboratory have shown 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 (Brady and Duncan,

1994b). It was apparent that the cell wall was the major, if not sole contributor to heavy metal accumulation at low ambient heavy metal concentrations.

Preliminary 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.

The aim of this study was to confirm previous findings in our laboratory concerning the mechanism of bioaccumulation of Cu^{2+}, Co^{2+} and Cd^{2+} by the cell walls/ cell wall components of *S. cerevisiae* by isolation of cell wall components and also by use of infra-red spectroscopy. Use of acid and alkali treatments for the removal of metal ions from the cell wall components and the reuse of these compounds for bioaccumulation was also investigated.

2.2 MATERIALS AND METHODS

2.2.1 YEAST CELL DISRUPTION

Before disruption, fresh bakers yeast (*S. cerevisiae*) was washed three times with Milli-Q (ultra-pure) water. Homogenization of the washed yeast cells was carried out at the Microbiology Department of the University of the Orange Free State using a Braun homogenizer. Cell counts using a Zeiss phase contrast microscope gave an average value for cell lysis of 73%. The cell debris was removed through centrifugation and the remaining material freeze-dried. To separate the cell walls from the intact cells, the solution was centrifuged at 250 g x 5 min. The pellet obtained by recentrifuging the supernatant (4 000 g x 10 min) was retained and freeze-dried.

2.2.2 CHEMICAL ISOLATION OF CELL WALL COMPONENTS

Glucan

Using the method of Northcote and Horne (1952) lyophilized cell wall material was digested with 3% (w/v) aqueous NaOH (90°C, 6 hrs). The insoluble residue obtained after centrifugation (3 000 g x 10 min) was extracted with 0.5N acetic acid (75°C, 6 hrs). The pellet was washed with ethanol and ether to remove the lipids. The remaining precipitate was dialysed (2 x 4 hrs, 4°C) and freeze-dried to obtain the glucan fraction.

Mannan

The alkali soluble supernatant obtained after NaOH treatment of the cell walls, was acidified, and the mannan precipitated with 4 volumes of ethanol. The precipitate was separated from the supernatant, resuspended in water and reprecipitated. The white solid was subsequently washed with ethanol and ether, dialysed following the normal procedure and freeze-dried overnight.

Chitin

Following the method of Muzarelli, *et al* (1980), yeast cell walls were demineralized with 5% HCl (5 hrs) using 11.8 : 1 (w/w) of HCl to dry weight of yeast cell walls. The pH of the sample was increased to neutrality by washing with water. After deproteination with a 2% w/v NaOH solution (65°C, 2 hrs) the sample was rewashed to neutrality. Included in the precipitate could have been some chitosan, possibly formed during NaOH treatment. The chitin/chitosan pellet dialysed, followed by freeze-drying overnight.

2.2.3 COMPOUND ANALYSIS

Sample Preparation

Components were hydrolysed using hot acid : 1 mg chitin was hydrolysed in 1 ml 4M HCl (100°C, 4 hrs) and 1 mg mannan and 1 mg glucan were each hydrolysed in 1 ml 2M HCl (100°C, 6 hrs).

Protein Assay

The Folin-Ciocalteau assay procedure was used (Clark and Switzer, 1977). A standard curve was prepared using a 1 mg/ml bovine serum albumin (BSA) standard. Cell wall components

were anlaysed using 3 x 0.1 ml aliquots of the prepared samples. All absorbance readings were made at 500 nm against a blank.

2.2.4 METAL UPTAKE STUDIES

To measure the uptake potential of each of the cell wall components, a buffered solution (5 mM Pipes/TMAH buffer, pH 6.5) of each component was dialysed against a metal containing solution. A multi-compartment dialysis apparatus based on that first used by Marrack and Smith in 1932, was used. The apparatus consisted of two perspex halves with five 1.2 ml chambers each. These halves were screwed together with a single layer of dialysis tubing between them to form five 2.4 ml chambers each bisected by a dialysis membrane. Two small perspex balls were used to act as stirrers in each chamber (See Figure 2.1).

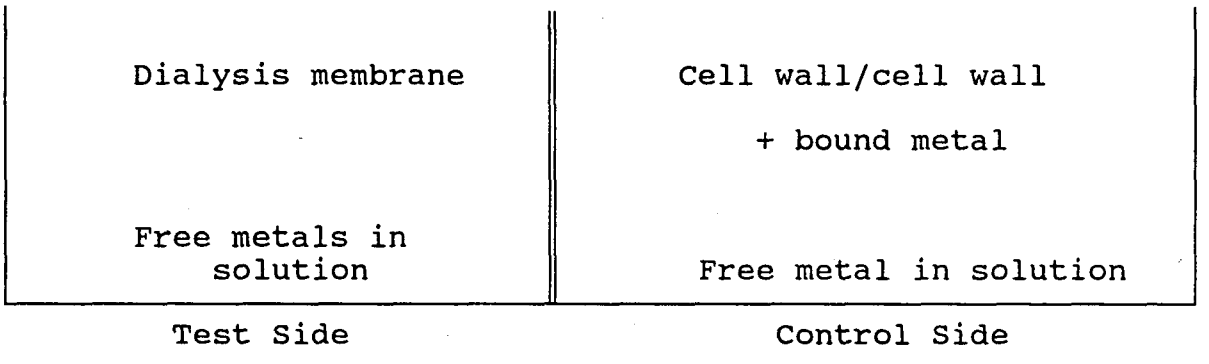


Figure 2.1: Diagramatic representation of the membrane dialysis apparatus

In order to form an equilibrium, the metal ions on the test side moved across the dialysis membrane and once in the vicinity of of the cell wall component, some metal ions bound to the cell wall component molecules. This metal binding in turn caused more metal ions to cross the dialysis membrane until a final equilibrium was reached. By determining the amount of free metal remaining in solution at the end of the experiment, it was possible to determine the amount of metal which had bound to the component in question :

i.e. BOUND METAL = TOTAL METAL CONC. - FREE METAL CONC.

Range Finding Experiments

Range finding experiments were carried out in order to determine the concentration of metal solution to be used, and the duration for which to run the experiments.

For determination of the metal concentration to be used, 5 mM Pipes/TMAH buffer was dialysed against metal solutions with concentrations ranging from 1 mM to 10 mM. Readings were taken of the metal concentration on either side of the dialysis membrane after 5 hrs using the AA spectrophotometer. The concentration used for subsequent experiments was that at which equilibrium was first observed, namely 4 mM for copper and cadmium, and 2 mM for cobalt.

To determine the time needed for maximum efficiency of the dialysis system; a metal solution of the previously determined concentration was dialysed against 5 mM Pipes/TMAH buffer. Readings were taken of the metal concentration on either side of the dialysis membrane at time = 2.5; 3; 3.5; 4; 4.5; 5; 5.5 hours. The time used for subsequent experiments was that at which equilibrium was first observed, namely 4 hrs for copper and 3 hrs for cobalt and cadmium.

Metal Uptake Experiments

A 1 mg/ml solution of whole cell wall, and of each of the isolated cell wall components in 5 mM Pipes/TMAH buffer was dialysed against the determined concentration of metal solution in Milli-Q water for the specific time determined in the range finding experiments. The apparatus was placed in a shaking water bath (22°C). At the end of the experiment the metal concentration remaining on the test side was determined. After removing the cell wall/cell wall component material from the control side, the apparatus was then filled with Milli-Q water and shaken in the water bath for a period of time equal to that of the experiment. Metal concentrations resulting from the release of metal ions which had complexed with the apparatus during the uptake experiment, could then be measured (these concentrations were found to be negligible and were omitted from calculations). These experiments were carried out on five samples of cell wall and five samples of each cell wall component, for each metal tested.

2.2.5 METAL REMOVAL

To remove the bound ions from the cell wall/cell wall components, acid and alkali treatments were used. Four samples of each buffered solution containing cell wall/cell wall component was centrifuged down (3 000 g x 10 min). The supernatant contained the unbound (free) metal left in solution. The pellet contained the cell wall/cell wall component with the bound metal. Two of each of these pellet samples were subjected to a treatment of 1 ml of 2N HCl for 2 hrs. The remaining two pellets were treated with 1 ml 2N NaOH for 2 hrs. All of the solutions were then spun down (3 000 g x 20 min). The supernatant then contained the previously bound metal which had been released by the acid/alkali treatment. The pellet contained the cell wall/cell wall component which was then washed with Milli-Q water and resuspended in 5 mM Pipes/TMAH buffer. Both the unbound metal concentration and the bound metal concentration from the supernatants obtained in the above experiment, were determined using an AA spectrometer.

2.2.6 METAL REUPTAKE

A second metal uptake experiment was carried out as described previously using copper and the cell wall/cell wall components which had had initially bound copper removed by the acid/alkali treatments. The metal ions from this reuptake experiment were removed as described above.

2.2.7 METAL ACCUMULATION STUDIES USING INFRARED SPECTROSCOPY

The respective samples (cell walls; cell wall components) were suspended in 5 mM Pipes/TMAH buffer (pH 6.5) to obtain a 1 mg (dry weight)/ml solution. Four 10 ml volumes of each of the samples were pipetted into separate flasks, and whilst one of these remained the control, the other three were exposed to heavy metal ions. 10 ml of 10 mM solutions of $CuCl_2$, $CoCl_2$ and $CdCl_2$ were pipetted into the respective flasks to obtain a final metal ion concentration of 5 mM.

Following incubation for 5 hours at 25°C (with shaking), the test and control fractions were

centrifuged (5 000 g x 20 min). The pellet was washed using Milli-Q water and resuspended in a minimal amount of Milli-Q water. Similar to the method of Kuyucak and Volesky (1989) this paste was evaporated to dryness at 70°C.

The infrared spectra of the various samples were determined as alkali pellets. Approximately 1 mg of the dried yeast sample and 100 mg of anhydrous KBr were mixed, and ground to a fine powder using as agate mortar and pestle. This powder was pressed into discs to obtain a clear window (Crowe and Crowe, 1986).

The infrared (IR) spectra of the native and treated *S. cerevisiae* cell wall and cell wall components were measured using a Perkin Elmer 180 Infrared Spectrometer, coupled to a digital plotter. To obtain information regarding the nature of the chemical interactions between the divalent cations and the yeast cell wall and cell wall components, the shift of the spectra in the 4 000 - 400 cm^{-1} region was measured (Tsezos and Volesky, 1982a, 1982b, Tsezos, 1983, Kuyucak and Volesky, 1988).

2.3 RESULTS

2.3.1 COMPONENT ISOLATION

The percentage yields for the isolated components of the yeast cell wall were determined (See Table 2.2).

Table 2.2: Percentage yields of cell wall components

Component	Starting Mass (mg)	Mass After Isolation (mg)	% Yield
Chitin	4 000	370.0	9.25
Glucan	6 000	237.5	3.96
Mannan	6 000	17.5	0.292

2.3.2 COMPOUND ANALYSIS

The values obtained for the protein concentrations in each of the isolated components are given in Table 2.3. According to the values given by Northcote and Horne (1952) (Table 2.1), protein and carbohydrate are the two main components of the yeast cell wall, with the remaining compounds (phosphorus, lipids, etc.) constituting approximately 10% of the yeast cell wall weight. Having determined the mean protein concentration in each of the components it was possible to calculate the carbohydrate content using the following equation:

TOTAL MASS = PROT. MASS + CARBOHYDRATE MASS + 10% TOTAL MASS

Table 2.3: Protein and carbohydrate concentrations and % (by weight) in each of the isolated components

Component	[Prot] (mg/mg yeast)	% Protein	[CHO] (mg/mg yeast)	% CHO
Chitin	0.19 ± 0.01	19	0.71	71
Glucan	0.02 ± 0.0	2	0.88	88
Mannan	0.22 ± 0.02	22	0.68	68

2.3.3 METAL UPTAKE

The bound metal concentrations calculated as described in the material and methods are shown in Table 2.4 and Figure 2.2.

Table 2.4: Metal uptake by cell wall/cell wall components

Component	Bound Metal Conc/mg Component (μmol/mg)		
	Copper	Cobalt	Cadmium
Cell Walls	0.24 ± 0.17	0.13 ± 0.05	0.17 ± 0.12
Glucan	0.36 ± 0.17	0.32 ± 0.21	0.34 ± 0.11
Mannan	1.12 ± 0.11	0.43 ± 0.10	0.39 ± 0.09
Chitin	0.60 ± 0.20	0.32 ± 0.27	0.46 ± 0.04

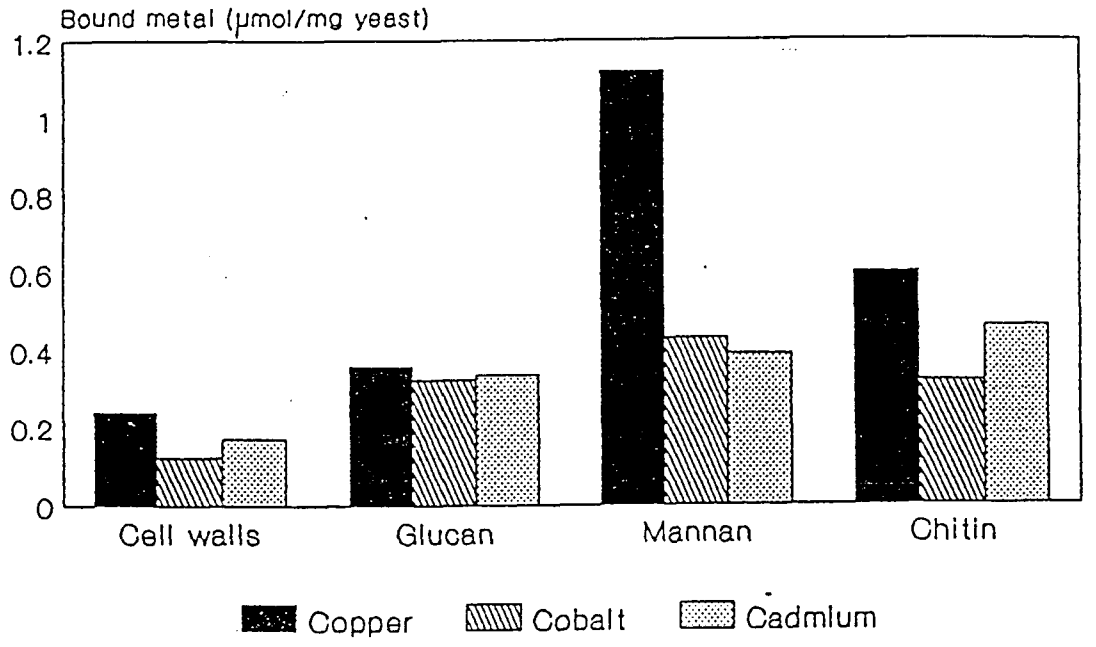


Figure 2.2: Metal uptake by yeast cell wall components

To determine the affinity constants (Ka) of the components for the various metals, Scatchard plots (Segel, 1976) were constructed *viz.* bound/free metal conc/vs/ bound metal conc (Figures 2.3, 2.4 and 2.5). The slopes of these graphs represent Kd, and the Ka values are calculated as the inverse of these values (Table 2.5). A graphic representation of the different affinities of the cell wall and cell wall components for the metal ions is given in Figure 2.6.

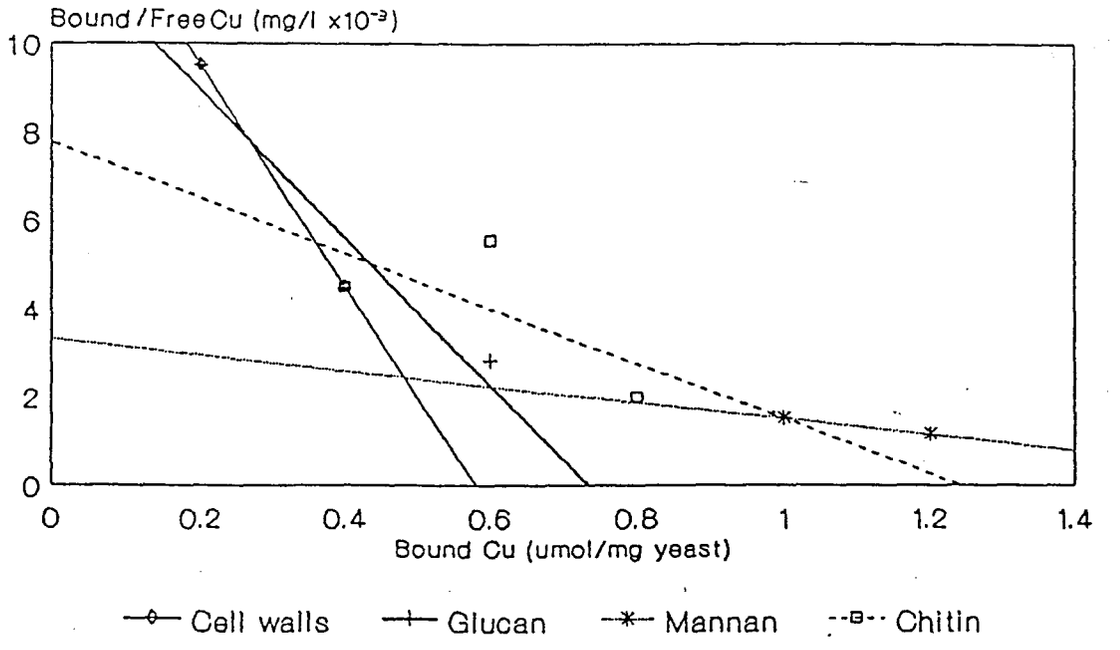


Figure 2.3: Scatchard plot of Cu binding to yeast cell wall components

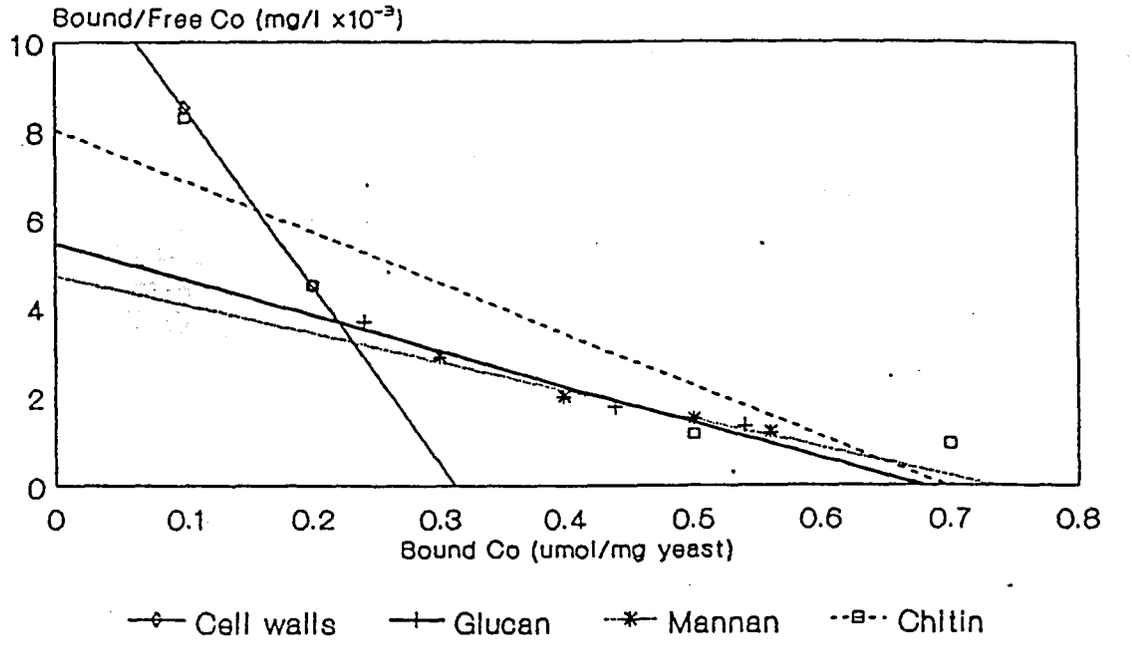


Figure 2.4: Scatchard plot of Co binding to yeast cell wall components

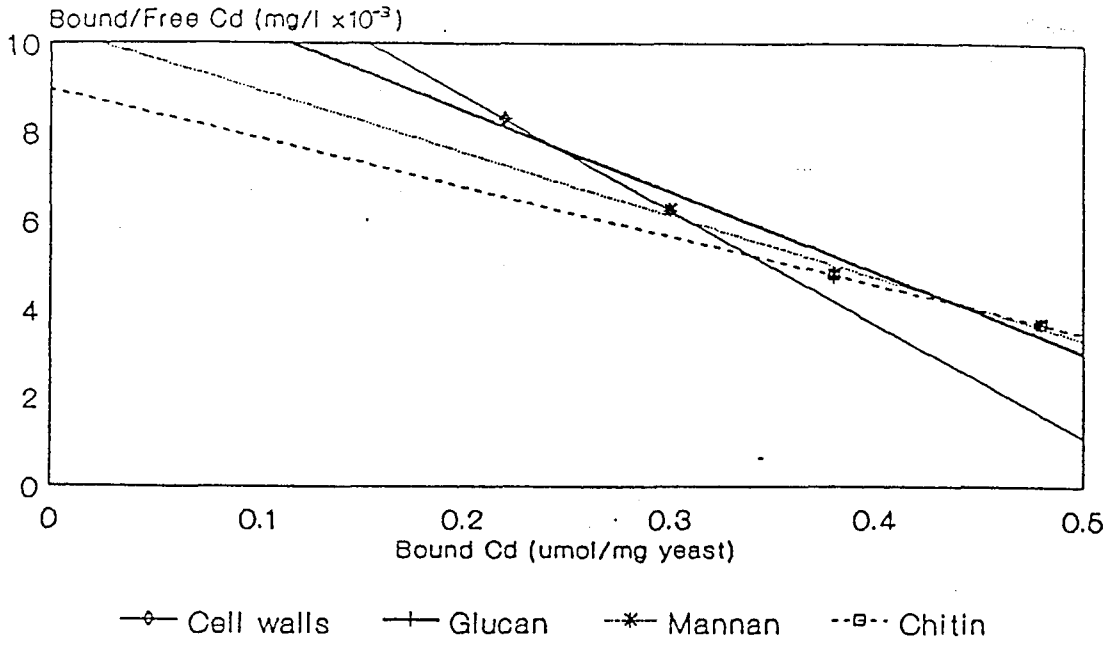


Figure 2.5: Scatchard plot of Cd binding to yeast cell wall components

Table 2.5: Affinities of cell wall/cell wall components for metal ions (Ka in ℓ/mol and Kd in mol/ℓ

Component	Copper		Cobalt		Cadmium	
	Kd	Ka	Kd	Ka	Kd	Ka
Cell walls	25.1	0.04	38.0	0.03	25.6	0.04
Glucan	16.7	0.06	7.8	0.13	17.7	0.06
Mannan	1.8	0.55	6.2	0.16	14.2	0.07
Chitin	8.9	0.11	12.3	0.08	11.0	0.09

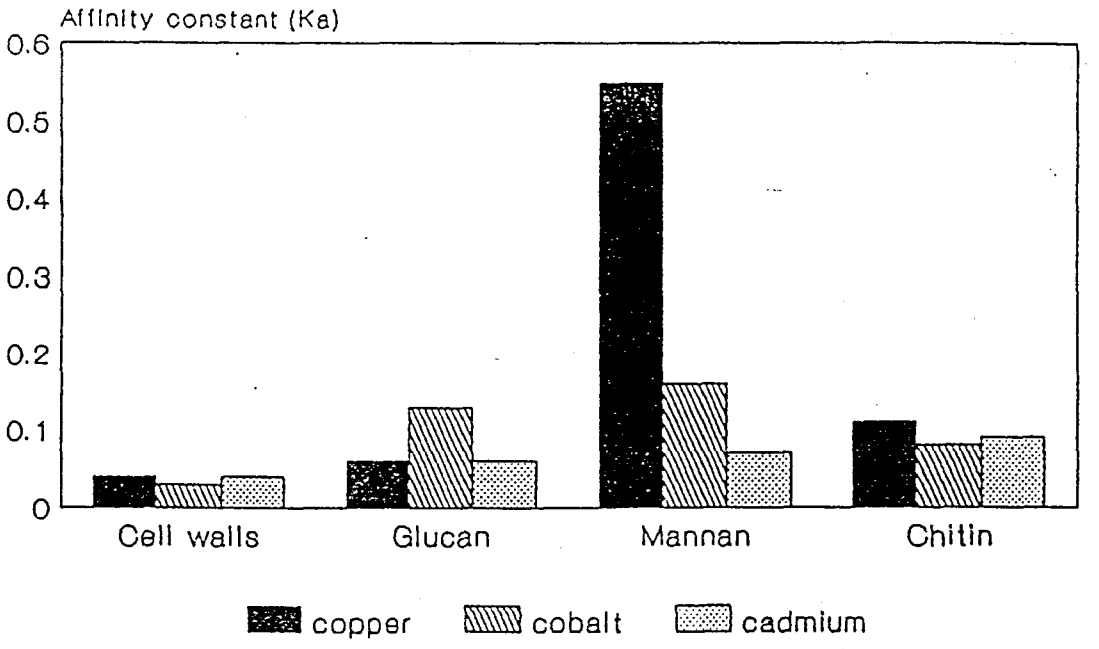


Figure 2.6: Affinity of yeast cell wall components for metal ions

2.3.4 METAL REMOVAL

The concentrations of metal ions removed by the acid/alkali treatments to which the cell wall and cell wall components were exposed, are given in Tables 2.6, 2.7 and 2.8. Generally the results of the acid treated cell walls and cell wall components mirror those obtained previously. The values obtained from alkali treated material were, however, much lower than those values obtained previously, but followed a similar trend (Table 2.4).

Table 2.6: Released Cu^{2+} conc. after acid/alkali treatment

Component	Cu^{2+} Conc. (μmol/mg) [Acid treated]	Cu^{2+} Conc. (μmol/mg) [Alkali treated]
Cell walls	0.30 ± 0.0	0.15 ± 0.07
Glucan	0.50 ± 0.0	0.15 ± 0.07
Mannan	1.03 ± 0.25	0.45 ± 0.07
Chitin	0.58 ± 0.10	0.15 ± 0.07

Table 2.7: Released Co^{2+} conc. after acid/alkali treatment

Component	Co^{2+} Conc. (μmol/mg) [Acid treated]	Co^{2+} Conc. (μmol/mg) [Alkali treated]
Cell walls	0.30 ± 0.0	0.26 ± 0.04
Glucan	0.53 ± 0.04	0.18 ± 0.14
Mannan	0.04 ± 0.07	0.15 ± 0.0
Chitin	0.25 ± 0.0	0.15 ± 0.0

Table 2.8: Released Cd^{2+} conc. after acid/alkali treatment

Component	Cd^{2+} Conc. (μmol/mg) [Acid treated]	Cd^{2+} Conc. (μmol/mg) [Alkali treated]
Cell walls	0.25 ± 0.0	0.08 ± 0.0
Glucan	0.42 ± 0.18	0.06 ± 0.03
Mannan	0.25 ± 0.06	0.04 ± 0.0
Chitin	0.29 ± 0.06	0.04 ± 0.0

2.3.5 METAL REUPTAKE

Copper reuptake by acid/alkali treated cell wall/cell wall components showed that acid treated samples bound less metal than alkali treated samples. Table 2.9 shows that reuptake of copper by alkali treated material was even greater than the initial uptake, with the exception of mannan (Figure 2.7).

Table 2.9: Initial and reuptake values of copper by cell wall and cell wall components (alkali treated material)

Component	Bound copper conc/mg component (μmol/mg)	
	Initial Uptake	Reuptake
Cell Walls	0.24 ± 0.17	1.2 ± 0.28
Glucan	0.36 ± 0.17	1.3 ± 0.42
Mannan	1.12 ± 0.11	1.0 ± 0.28
Chitin	0.60 ± 0.20	1.3 ± 0.14

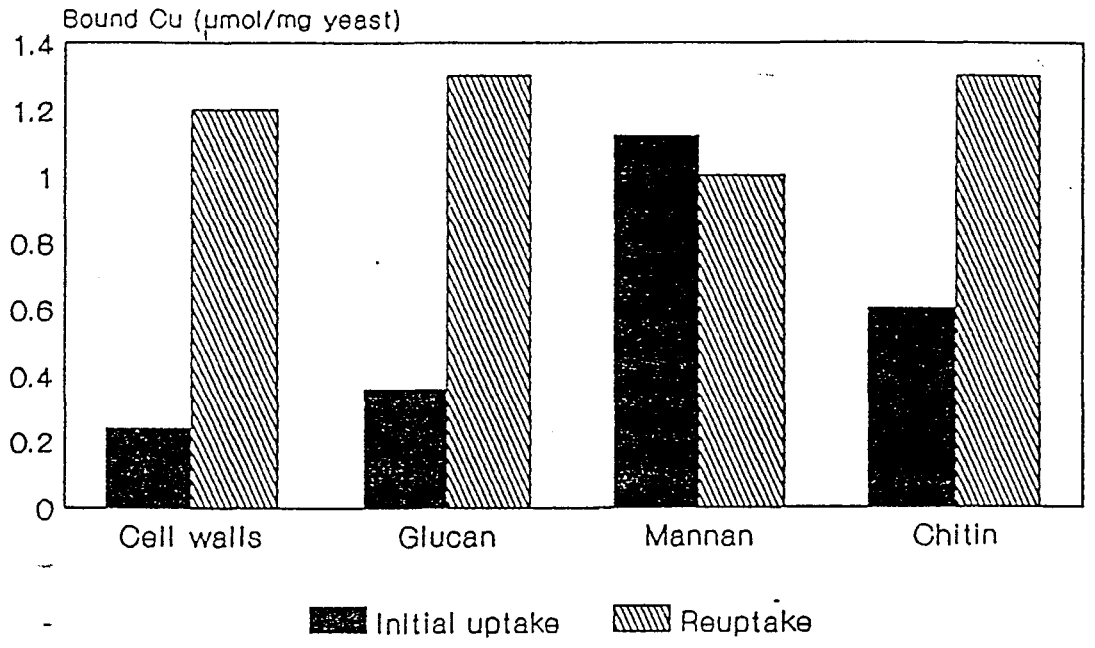


Figure 2.7: Reuptake of Cu ions by alkali treated yeast cell wall components

2.3.6 BINDING PATTERNS OF Cu^{2+}, Co^{2+} and Cd^{2+} TO YEAST CELL WALLS

Cu^{2+}, Co^{2+} and Cd^{2+} differed only regarding their intracellular bioaccumulation patterns. Infrared spectra (Fig. 2.8 a-d) exhibiting the relationship between isolated yeast cell walls and the respective metal cations show the complexity of the binding patterns. The three metal ions have almost identical binding patterns to the cell wall, and affect primarily the 2° amides. Glucan and chitin, exhibited the same trend regarding metal ion accumulation. However, the spectra heavy metal exposed mannan fractions differed from those of pure mannan (data not shown).

Differences occurring between the three cations and the cell wall material were limited to the amounts of the metal cations bound to the cell wall components which were indicated by the differing peak heights and intensities.

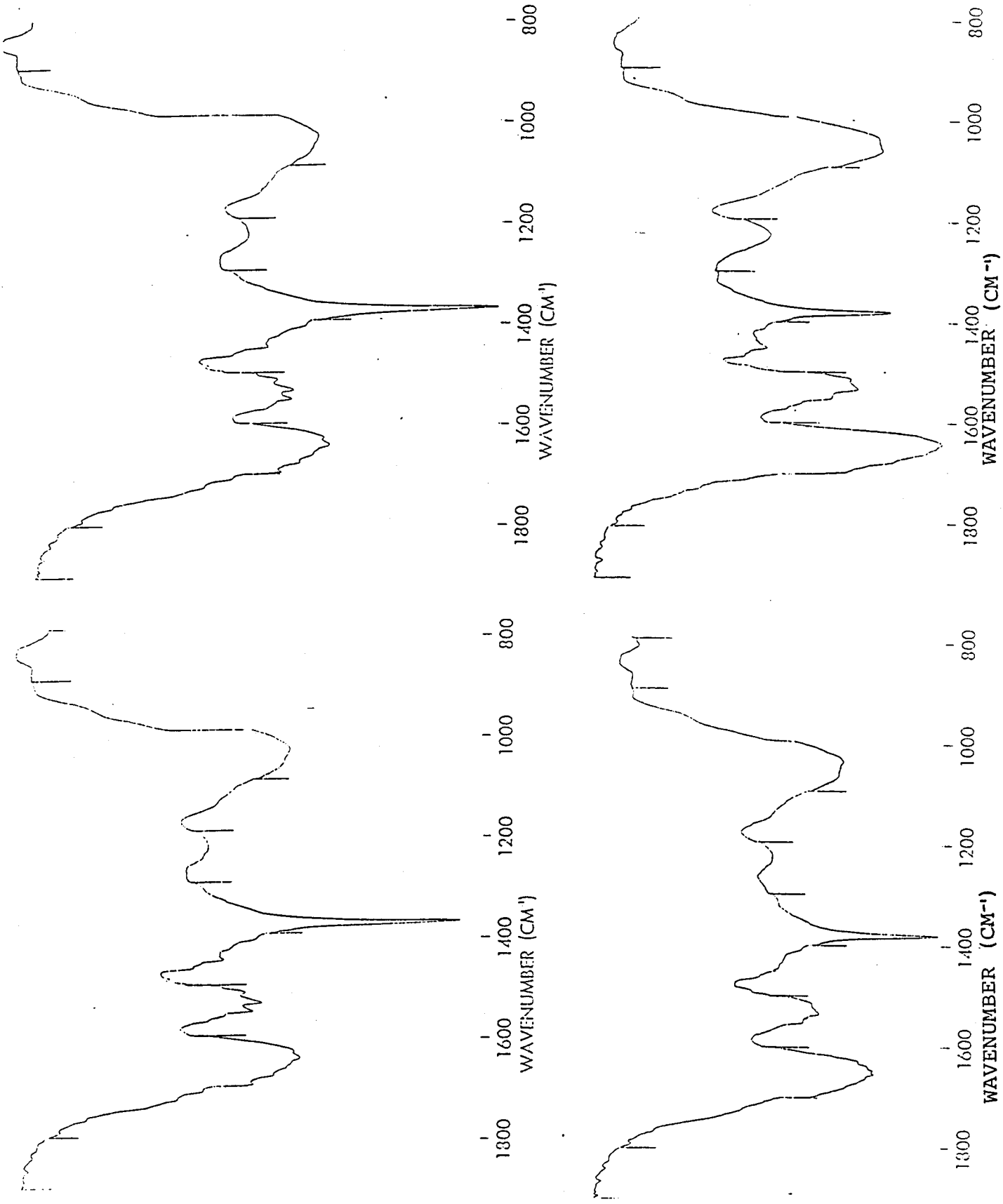


Figure 2.8: Infrared spectra of the binding patterns of metal ions to yeast cell walls (YCW), (a) native cell wall material, (b) Cu^{2+}-YCW, (c) Co^{2+}-YCW and (d) Cd^{2+}-YCW

2.4 DISCUSSION

2.4.1 ISOLATION AND ANALYSIS OF COMPONENTS

Yields of the various components isolated (Table 2.2) were relatively low compared to those values obtained in previous studies in this laboratory (Brady, 1992). The chitin component was shown to contain a significant amount of protein which should theoretically have been removed during the deproteinization step in the isolation procedure. The glucan component showed a characteristically low protein to carbohydrate ratio, and the isolation procedure therefore appears to have yielded a fairly pure product. The protein content of the mannan is high due to the existance of the mannan-protein complex in the yeast cell wall (Table 2.3).

The acid and alkali fractionation techniques used for component isolation served to break the glycosidic links of the polysaccharide components and the peptide bonds of the proteins in the cell wall which are thought to bind together the various cell wall polysaccharides (Bartnicki-Garcia and McMurrough, 1971). The acid and alkali techniques should also have broken some of the bonds of the mannan-protein complex and the protein content of the mannan component may therefore be unrealistically high.

2.4.2 METAL UPTAKE

One of the aims of this study was to establish if the various cell wall components had different affinities for different metals and if specific components showed greater affinities for specific metals. In the metal uptake experiments it was assumed that the formation of a metal-component complex at one site did not alter the affinity of the other sites for the metal ions, although steric hindrance of binding groups by bound metal ions could possibly influence this assumption. The results obtained from these experiments show the following general trend (Table 2.4, Figure 2.2) :

- In all cases the isolated components bound more metal than the whole cell walls. This can be attributed to steric hindrance i.e. once isolated, the groups available to bind the metal cations become exposed, whereas in the whole cell wall, these groups are unavailable for binding (Muraleedharan *et al*, 1990).

- In all cases mannan and chitin components bound more metal than the glucan component. This can be expected if one considers the reactive groups available for binding in each of the components. The mannan component has hydroxyl groups, phosphorus groups, and generally a high proportion of hydroxyl and dicarboxylic amino acids (Phaff, 1971). The chitin component has hydroxyl groups, nitrogen groups and oxygen groups. The glucan component has only hydroxyl groups.

Oda *et al*, (1988) digested the cell walls with pronase so as to decrease their nitrogen content, and observed a decrease in cadmium binding. They therefore concluded that nitrogen groups are actively involved in cadmium binding and this would explain the high amount of cadmium bound by the chitin component in the metal uptake experiments.

It would seem that several metals share the same binding sites since Nakajima *et al*, (1986) found that when *S. cerevisiae* was exposed to a mixture of metals (Mn, Co, Ni, Cu, Zn, Cd, Hg, Pb, U) the cells bound 2.82×10^{-4} mol/g of yeast whereas on exposure to cobalt alone, the value was 0.99×10^{-4} mol/g of yeast. The authors suggested that this is due to interionic competition during the bioaccumulation process.

The Ka values obtained (Table 2.5) from the Scatchard plots (Figures 2.3, 2.4 and 2.5) were used to produce Figure 2.6, showing the affinity of yeast cell wall components for the various metal ions. This graph roughly resembles the data in Figure 2.2 showing the uptake of metal ions by the components. One unexplained discrepency in the graphs is the high affinity which the glucan component has for cobalt and the relatively low uptake value for this metal by glucan.

Apart from the high binding of cadmium by chitin, it was generally observed that the copper was bound more than the cobalt which was bound in approximately the same amount as cadmium. This may possibly be explained by the fact that copper is the smallest of the three cations used and consequently has greater access to the functional binding groups on the cell walls and cell wall components.

2.4.3 METAL REMOVAL

As shown in Tables 2.6, 2.7 and 2.8, the acid treatment to which the components were subjected was more effective for removing metal ions than the alkali treatment. Metal removal by acid treatment was generally two-fold greater than removal by alkali treatment. The acid treatment displaces bound metal ions by substituting protons for the metal cation. These metal removal treatments may have resulted in substantial loss of material, particularly the alkali treatment which may have caused solubilization of some components e.g. mannan. In the case of the cell wall samples, both the acid and the alkali treatments caused breakdown of the structure which allowed for exposure of groups involved in metal binding.

2.4.4 METAL REUPTAKE

Copper reuptake by alkali treated cell wall/cell wall components was greater than for acid treated cell wall/cell wall components (Table 2.9). This may be explained by the fact that the hydroxyl groups present during the alkali treatment are capable of binding to the component and acting as additional binding sites during the reuptake experiments. Reuptake by the cell wall was very much greater than the initial uptake due to the exposure of new binding groups as explained above. Figure 2.7 shows that the glucan and chitin components also exhibited increased copper reuptake whereas the mannan component showed decreased reuptake. The initial and reuptake values may however not be comparable due to the loss of material during the experimental procedure and due to the small sample number used in the reuptake experiments.

2.4.5 BINDING PATTERNS OF HEAVY METALS TO CELL WALLS

Of the isolated cell wall components mannan has proven to be the predominant metal accumulator. This was substantiated by IR spectra obtained in the present study. IR spectra also indicated similar interactions of Cu^{2+}, Co^{2+} and Cd^{2+} with isolated yeast cell walls. Only minor differences could be detected between the spectra of native cell wall material and those exposed to metals. The majority of these differences were accounted for by differences in the peak height and intensity in the 1300-1400 cm^{-1} and 1500-1400 cm^{-1} regions, which represent 2° amide and carbohydrate moieties of the cell wall. As the majority of protein

within the cell wall is found associated with the mannan polymer (mannanprotein), these spectra confirmed the superior binding properties of mannoprotein above glucan and chitin.

Thus it can be concluded that yeast cells are capable of accumulating heavy metal cations. The cell wall is the first cellular structure to come into contact with the metals present in the extracellular solution. All cations appear to bind to the wall in a similar fashion and only differ in the amounts bound.

CHAPTER 3

BINDING PATTERNS OF HEAVY METALS TO *S. CEREVISIAE* CELLS

3.1 INTRODUCTION

Microorganisms are capable of accumulating metals by a number of different processes, e.g. accumulation by transport, precipitation, biosorption to cell walls, entrapment in extracellular capsules and oxidation reduction reactions (Gadd, 1990a, 1990b). Some, or all of these processes are utilized by viable microorganisms during the accumulation of metal ions.

The present study utilized electron microscopy and X-ray analysis to elucidate the binding patterns of heavy metals to *S. cerevisiae* cells and to identify where different metal ions are localised in the cells.

3.2 MATERIALS AND METHODS

3.2.1 PRETREATMENT OF YEASTS

Commercial bakers yeast (*Saccharomyces cerevisiae*) was obtained from Anchor Yeast Industries (production strain withheld). The yeast cells had been grown aerobically in molasses wort and harvested in stationary phase. To remove impurities the yeast was washed three times (3 000 g x 10 min) with ultra-pure (Milli-Q) water.

3.2.2 INTRACELLULAR METAL ION ACCUMULATION AND X-RAY ANALYSIS

Metal Accumulation

Washed yeast cells were resuspended in a 5 mM Pipes/TMAH buffer (pH 6.5) in a 1:2,5 ratio (wet weight : ml buffer). 2 mol/ℓ stock solutions of the respective heavy metal salts, *viz.* $CuCl_2$, $CdCl_2$ and $CoCl_2$ were prepared. To the respective test fractions 20 ml of one of these salts was added. One 20 ml yeast-buffer fraction, the control, was not exposed to any of the abovementioned or other heavy metal cations.

Incubation of the fractions commenced for differing periods of time, *viz.* 1, 2, 5, 12 and 24

hours. Incubation of these solutions was conducted in a waterbath with shaking at 25°C. On completion of incubation, the yeast suspensions (both test and control fractions) were centrifuged (3 000 g x 10 min). The pellet was air dried for 5 min prior to being mixed with warm 6% agar and allowed to set.

3.2.3 EMBEDDING PROTOCOL

Blocks of agar (2 mm^3) were removed from the agar plates and placed into labelled specimen tubes containing cold 2.5% glutaraldehyde in 0.1 M phosphate buffer. Primary fixation of the sample occurred overnight. The blocks were subsequently washed three times with cold 0.1 M phosphate buffer.

No secondary fixation protocol was observed as the secondary fixative osmium tetroxide (OsO_4) caused elemental conflicts in the energy dispersive X-ray spectra of copper and other ions.

The washed samples were dehydrated using an alcohol gradient (30 - 100% ethanol). Following dehydration, the infiltration procedure could continue. The initial stage involved two 15 minute washes with propylene oxide (PO).

Infiltration with the embedding resin was gradual. On decanting off the second PO wash, the tubes were refilled with 75:25 PO : resin (Araldite) mixture. This was discarded after 60 minutes and replaced by a 50:50 PO : resin mixture for the same period of time. The procedure was repeated for a 25:75 PO : resin mix. From here the samples were transferred into tubes containing 100% pure resin (Araldite) and infiltration allowed to proceed overnight.

On comparison of infiltration the samples were transferred to capsules filled two thirds with pure resin. These were placed in a 60°C oven for 36 hours to enable polymerization to occur. (Embedding protocol adapted from Cross, 1987).

3.2.4 PREPARATION OF SECTIONS FOR TRANSMISSION ELECTRON MICROSCOPY (TEM) AND X-RAY DIFFRACTION ANALYSIS

The blocks were trimmed to the correct shape, a trapezium, prior to the cutting of ultrathin sections. All sectioning and trimming of the samples were conducted on a RMC MT-7 Ultramicrotome. Glass knives were prepared using a LKB knifemaker.

Ultrathin Sections for TEM

Ultrathin sections (100 nm) were cut from the pretrimmed block using a glass knife to which a plastic trough, filled with glass distilled water had been attached. The cut, 100 nm sections were collected by floating them onto 300 mesh copper grids, which were placed on filter paper to dry.

Ultrathin Sections for X-ray Analysis

Thicker sections were required for X-ray analysis. 250 nm thick sections were cut from the trimmed block, using a new knife. The sections when cut, formed a blue-green ribbon which was floated onto grids. Due to various elemental conflicts in the X-ray analysis spectra, not all the samples could be collected onto the same grid type. The $CuCl_2$ treated samples were collected onto 300 mesh gold grids and $CoCl_2$ samples onto wide slot formvar coated copper grids. $CdCl_2$ samples were collected onto formvar coated wide slot copper grids or 300 mesh nickel grids. Due to the presence of heavy metals in the staining solution, the sample sections to be analysed using EDAX spectra were not stained. Once dried, the grids were carbon coated.

Staining Techniques and TEM of Samples

Due to interferance with the detection of the heavy metal cation depositions within the cell, the X-ray analysis samples were not stained. The thin (100 nm) TEM sections were stained with saturated aqueous uranyl acetate (30 min) and lead citrate (5 min). Both staining procedures were followed by water washes.

Examination of the stained grids involved the use of a JOEL JEM 100 CK II Transmission Electron Microscope at a 80 kV acceleration voltage.

Energy Dispersive X-ray Analysis

The presence of internalized and intracellular metal ions in the yeast cells of the 250 nm sections were confirmed through examination of the carbon coated sections with a Phillips EM 420 Transmission Electron Microscope at an acceleration voltage of 120 kV. The sites of metal ion deposition, localization and patterns of ion exchange were determined using EDAX spectra obtained from an EDAX PV 9100 probe attached to the TEM. The lower and upper limits of the EDAX probe were set at 1 KeV and 20 KeV respectively, thereby excluding the cellular components : H, C, N, O from the spectra.

3.3 RESULTS

3.3.1 TEM OF Cu^{2+}, Co^{2+} AND Cd^{2+} LOADED CELLS

The morphological consequences of Cu^{2+} accumulation on the yeast intracellular structure are shown in Figure 3.1 (encircled areas indicate metal accumulation). The micrographs of the metal exposed cells illustrate the internalization and accumulation of metal cations. Enlarged centrally located electron dense bodies, the vacuoles, were present in Cu^{2+} loaded cells. Deposition of the metal ions within the vacuole appeared to be time dependent. Relatively few or no electron dense depositions appeared in the 1 hr samples. In comparison, the vacuole appeared to have a limit to the amount of Cu^{2+} it could accumulate. As seen from Figure 3.1 (c-e) the size of the vacuole remains relatively constant from 5 - 24 hrs.

The metal cations also appeared to accumulate as small bodies within the cell. This was demonstrated most clearly by Co^{2+} and Cd^{2+} uptake (Figures 3.2, 3.3). It is uncertain whether the presence of these bodies indicates accumulation of metal ions in smaller vacuoles or alternatively accumulation of the metal ions as inclusions within the cytosol of the yeast cell.

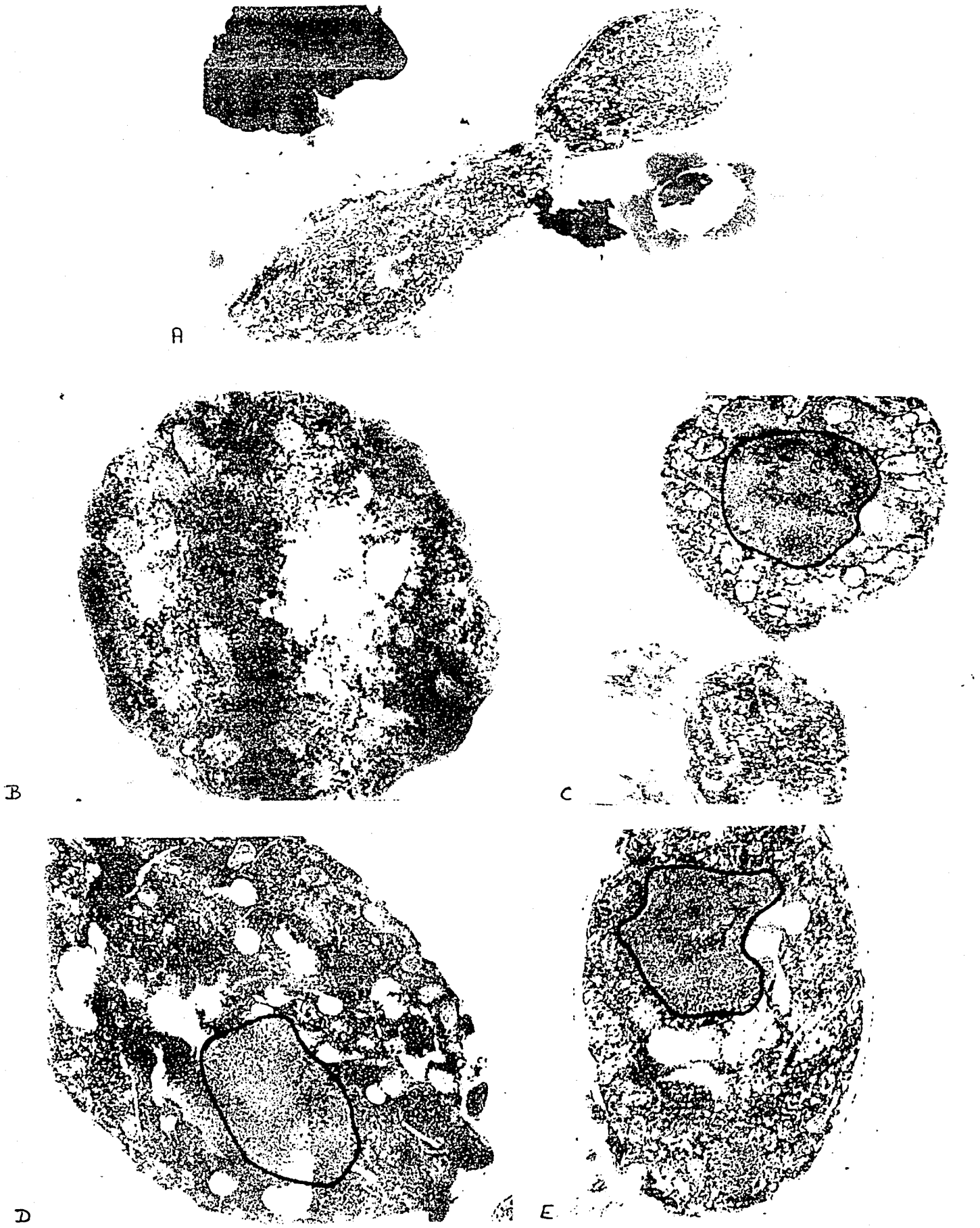


Figure 3.1: Electron micrographs of *S. cerevisiae* cells (a) native cells (10 000 x magnification); Cu^{2+} contact (b) 1 hr, (c) 5 hrs, (d) 12 hrs, (e) 24 hrs

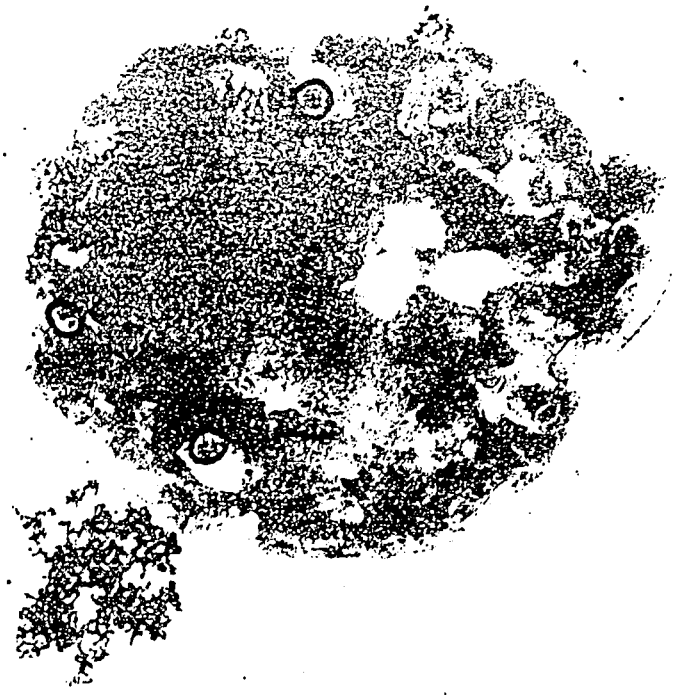


Figure 3.2: Electron micrograph indicating Co^{2+} accumulation as crystal formation within the cell (19 000 x magnification)

Figure 3.3: Cd^{2+} accumulation as crystals within the cell (19 000 x magnification)

Also apparent from this work was the effect of metal ion accumulation on the cellular tissue. The longer the time period that the yeast cells were exposed to the metal salts, the greater the extent of tearing of the cellular components during sectioning and slicing.

3.3.2 THE EFFECT OF HEAVY METAL CATIONS ON THE INTRACELLULAR ION COMPOSITION

Elements within the yeast cell only resulted in peak formation on the EDAX spectra when comprising 1% or more of the area under analysis. Restrictive criteria prevented quantitative analysis of metal ion concentration within the yeast cell; thus only qualitative results were obtained.

Intracellular ions present in unexposed cells and present in amounts equal to or greater than 1% of the cellular composition included : Na^{+}, Mg^{2+}, Si^{2+}, P^{-}, S^{-}, Cl^{-}, K^{+} and Ca^{2+} (Figure 3.4).

Complimentary to the TEM results, not all EDAX spectra of the respective heavy metal exposed cells yielded positive spectra, especially during the first hour. This implies that yeast cells may have accumulated the metals but in low quantities, as X-ray diffractive analysis of the intact cells often resulted in negative spectra for a specific ion, which was found to be located intracellularly (Table 3.1).

Table 3.1: Cellular ion composition of yeast cells exposed to Cu^{2+}, Co^{2+} and Cd^{2+} respectively. The incubation period was 1 hr and ambient heavy metal concentrations 1 $mol.l^{-1}$.

Control			Cu Cells			Co Cells			Cd Cells		
IC[a]	Cyt[b]	Vac[c]	IC	Cyt	Vac	IC	Cyt	Vac	IC	Cyt	Vac
Na	P	Na	Si	Si	Si	Na	P	Na	P	Na	Na
Mg	S	Mg	P	P	P	Mg	S	Mg	S	P	Mg
Si	Cl	Si	Cl	S	S	P	Cl	P	Cl	S	Si
P	K	P	Cu	Cl	Cl	S	Ca	S	K	Cl	P
S	Ca	S		Cu	Cu	Cl		Cl	Ca	Cd	S
Cl		Cl				K		K			Cl
K						Ca		Ca			Cd
Ca								Co			

a: IC - Intact Cells

b: Cyt - Random Location in Cytosol

c: Vac - Electron Dense Vacuole

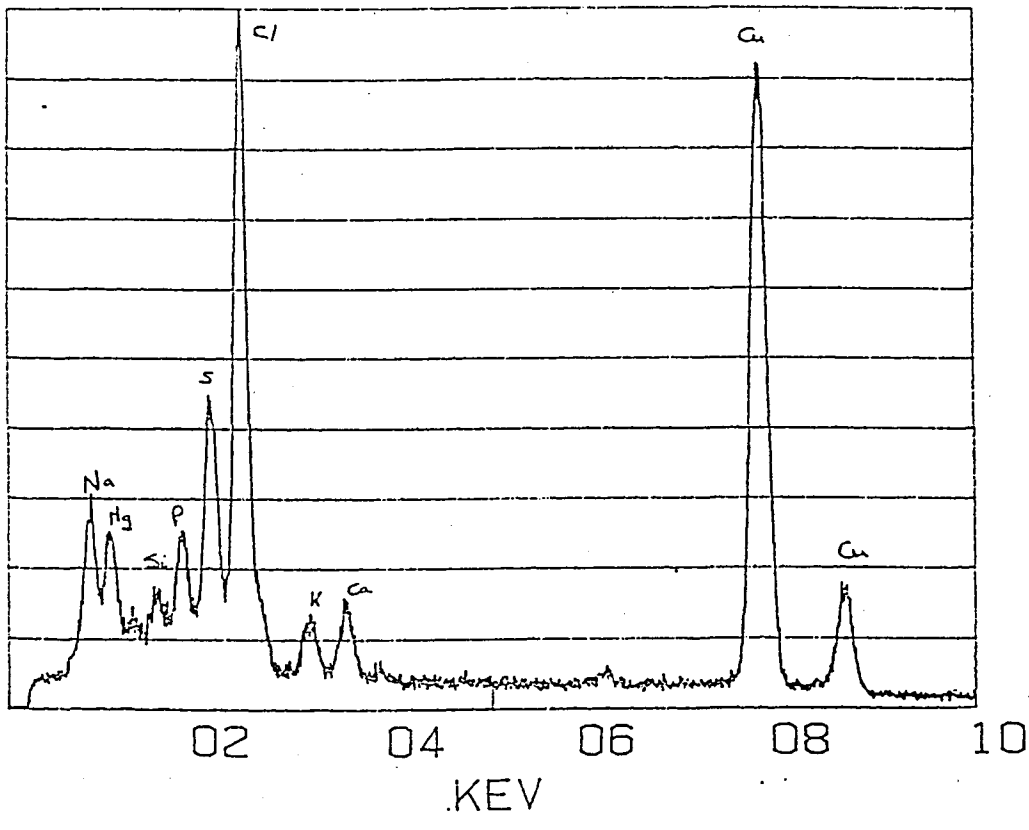


Figure 3.4: A typical EDAX system for native *S. cerevisiae* cells

Some yeast cells exhibited no heavy metal depositon at all, which implicates metabolically driven heavy metal accumulation and deposition within the cell, especially during the earlier stages of exposure. Exposure to 1M Cu^{2+}, Co^{2+} and Cd^{2+} for a period of time longer than 5 hrs resulted in the majority of cells having accumulated heavy metal cations.

Two types of heavy metal cation accumulation within the yeast cell occurred. Accumulation of Cu^{2+} and Cd^{2+} by yeast cells implicated an ion exchange mechanism. A decrease of cellular ions within the cells occurred on exposure to Cu^{2+} (Figure 3.5 a-c). On exposure to, and during the accumulation of Cd^{2+} the yeast cells exhibited a similar response (Figure 3.6 (a-c). However, in both instances Ca^{2+} ions remained within the cell at sufficiently high enough concentrations to be recorded.

This implies that either selective ion exchange occurred within the cells, i.e. Na^{+}, Mg^{2+} and K^{+} were eluted in preference to Ca^{2+} during heavy metal accumulation. Alternatively, the efflux of intracellular ions from the cell occurred at the same rate, yet higher initial levels of Ca^{2+} resulted in greater amounts of this ion remaining after 24 hrs.

Co^{2+} accumulation by the yeast cell exhibited a totally different pattern. No depletion of Na^{+}, Mg^{2+}, K^{+} and Ca^{2+} occurred over time (Figure 3.7 (a-c). Similarly, Okorokov (1985), discovered that Co^{2+} translocation into the yeast cell did not result in any K^{+} efflux from the cell.

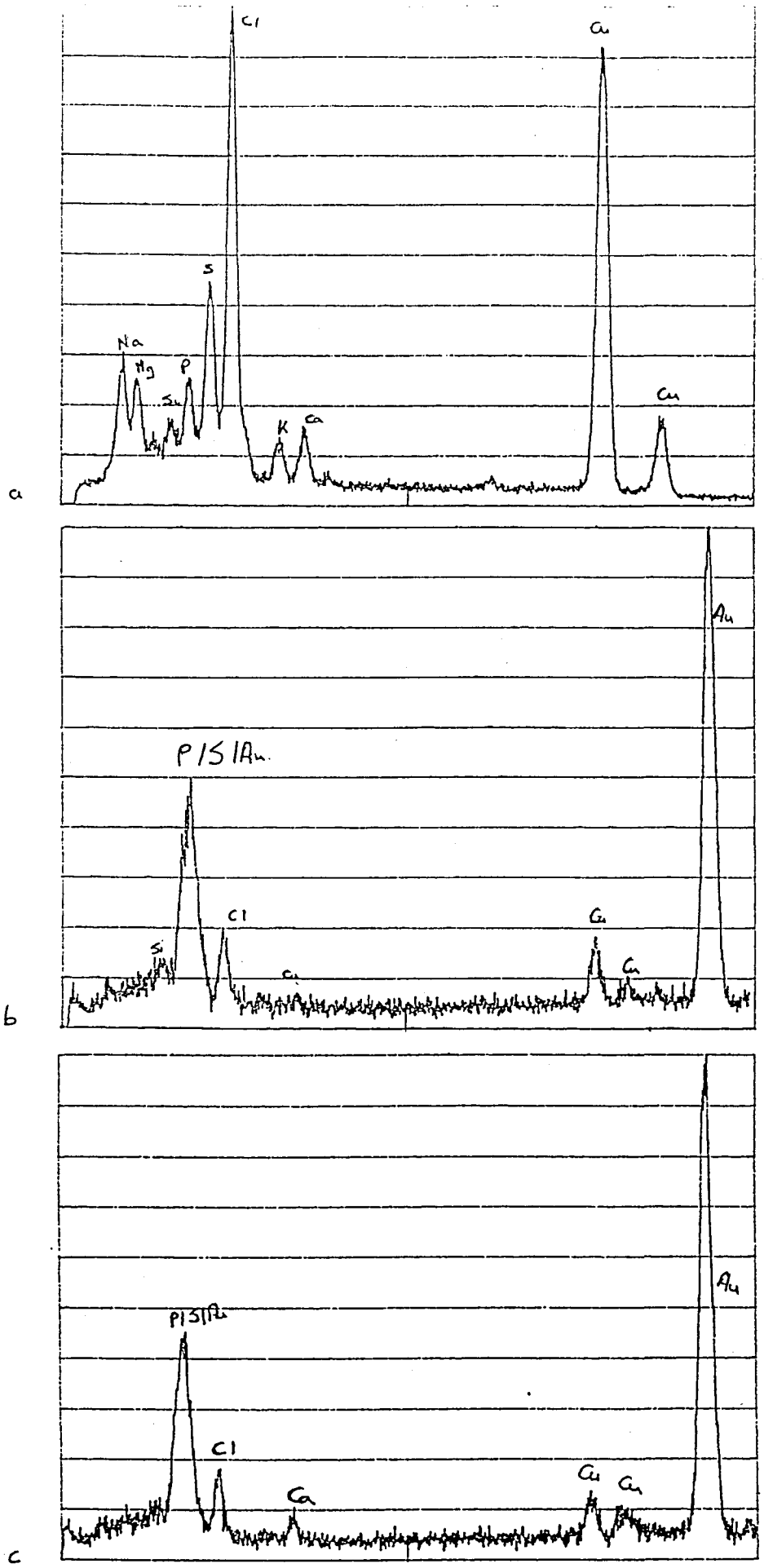


Figure 3.5: Ion exchange patterns in yeast cells on exposure to Cu^{2+}, (a) native cell, (b) 1 hr exposure, (c) 24 hrs exposure

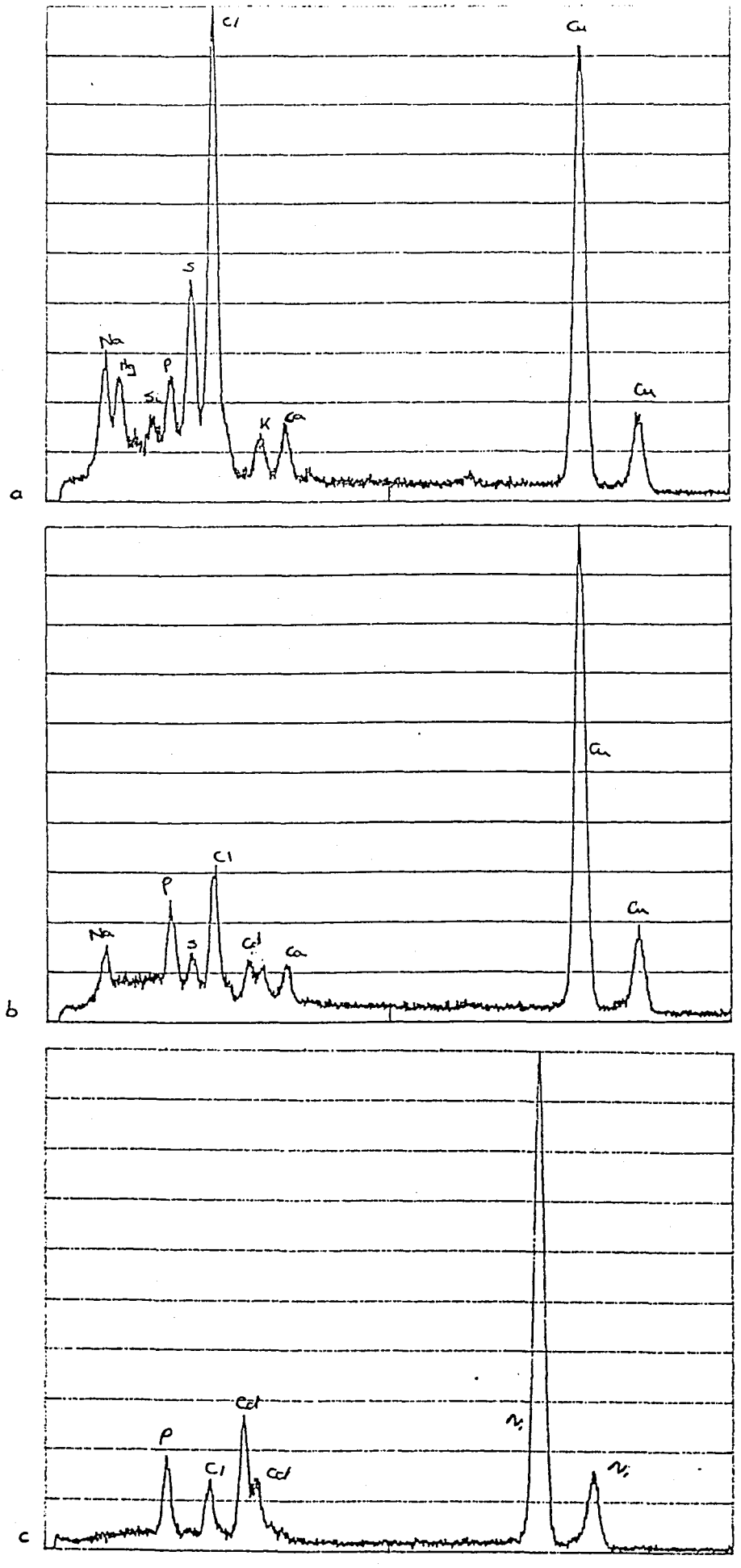


Figure 3.6: Intracellular ion composition of cells exposed to a cadmium containing solution, (a) native yeast cells, (b) 1 hr exposure, (c) 24 hr exposure. Cd^{2+} peaks mask the presence of K^{+}

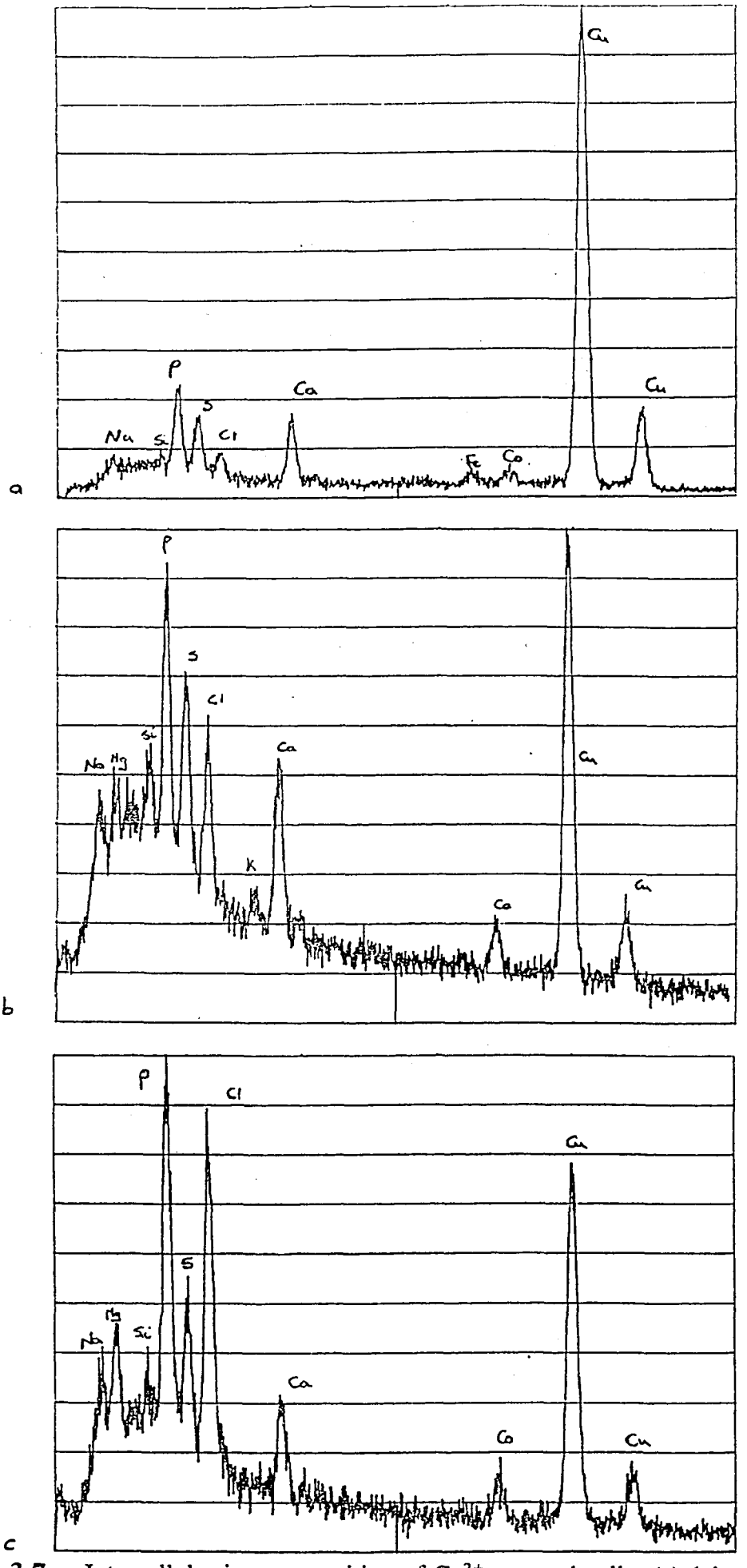


Figure 3.7: Intracellular ion composition of Co^{2+} exposed cells, (a) 1 hr, (b) 12 hrs, (c) 24 hrs

3.4 DISCUSSION

S. cerevisiae cells were capable of accumulation of metal ions from effluent solutions. A variety of mechanisms appeared to be responsible for the uptake and internalization of the metal ions. The amount of metal accumulated (mg metal/g yeast, wet weight) was dependent on the ambient metal concentration and was limited by low extracellular pH. Both the intracellular and extracellular pH affects the ion uptake by yeast. Extremes in pH affects the proton (H^+) gradient which is necessary for cation uptake (Jones and Gadd, 1990).

Uptake of cations from the surrounding environment into the cell is effected by a H^+/antiport system located within the plasma membrane and which is energetically powered by ATP hydrolysis (Klionsky, *et al*, 1990). Extremes in pH affect the proton (H^+) gradient. Evidence of the latter system was supplied by monitoring the pH during bioaccumulation. The longer the yeast cells were exposed to the effluent the higher the extracellular pH, suggesting a H^+ efflux from within the cells.

H^+ is one of the few ions involved in a ion exchange system between yeast cells and the surrounding environment. On exposure to concentrated Cd^{2+} and Cu^{2+} solutions, loss of intracellular ions from within the cell was observed. The rapid loss of K^+, Na^+ and Mg^{2+} from the cytosol was similar to the trends found by a number of others (Norris and Kelly, 1979, Okorokov, 1985) and which can occur via the ion exchange channels. Although heavy metals increase the permeability of the cellular membranes, e.g. Cd^{2+} which binds to organic ligands (Gadd and Mowll, 1983, Joho, *et al*, 1985, Ohsumi, *et al*, 1988) this did not appear to be the route along which intracellular efflux occurred. Exposure to Co^{2+}, a toxic divalent heavy metal cation, did not result in the efflux of intracellular cations. If this cation had mirrored the behaviour of similar cations by increasing membrane permeability, numerous intracellular ions would have leaked from the cell. Instead it appeared as though Co^{2+} had an inhibitory effect on the ATPase H^+/antiport system. Entry of Co^{2+} into the cytosol appeared to occur via the antiport system, after which the Co^{2+} blocked the channel preventing the release of any intracellular ions.

The effect of glucose pretreatment substantiated the theory that the majority of heavy metal cations enter the yeast cell via the antiport system and not through membrane lesions. Cells pretreated with glucose, an energy source, exhibited a superior ability to remove metal ions from solution. This increased ability of energized cells to accumulate additional metal cations is an indication of metabolic accumulation.

A further indication of metabolically driven accumulation of metal ions was provided by the fact that some yeast cells failed to accumulate any metal ions. If accumulation of metal ions was independent of metabolism, then all cells should have exhibited Cu^{2+}, Co^{2+} and Cd^{2+} deposits.

CHAPTER 4

VACUOLAR UPTAKE OF METAL IONS

4.1 INTRODUCTION

Fungal vacuoles are intracellular membrane bound organelles which are involved in a wide variety of essential physiological functions. They act as the primary storage site for important metabolites, including amino acids and inorganic phosphates. Many ions, including monovalent and divalent cations are located therein. Osmoregulation within the cell and the homeostatic regulation of the cytosolic pH are also functions of this organelle. Isolated vacuoles also contain a range of hydrolytic enzymes enabling endolytic macromolecular degradation (Matile, 1978, Boller and Wiemken, 1986, Wada, *et al*, 1987, Jones and Gadd, 1990, Klionsky, *et al*, 1990).

Likewise to the storage of nutrients and metabolites, the accumulation of metal cations within the vacuole requires a transport mechanism from the cytosol. It is essential to regulate the cytosolic ion concentration for several reasons : some ions, e.g. Co^{2+} and Cd^{2+} are potentially very toxic and have to be removed from the cytosol. Physiologically useful ions, including Cu^{2+}, Mg^{2+} and Ca^{2+} may become toxic at very high ion concentrations (Klionsky, *et al*, 1990).

According to Jones and Gadd (1990), the transport of both monovalent and divalent cations across the tonoplast is effected by enzymatic pumps, although precise clarity regarding the uptake properties of certain ions, including Fe, Co^{2+} and Ni^{2+} appears to be lacking (Klionsky, *et al*, 1990).

An electrochemical proton gradient, generated by H^{+}-ATPase energizes the transport of the majority of ions across the tonoplast. The vacuolar ATPase system is one of three types which is found to occur in the yeast cell (Okorokov, *et al*, 1983, Ohsumi and Anraku, 1983, Okorokov, 1985, Uchida, *et al*, 1985, Eilam, *et al*, 1985, Jones and Gadd, 1990). The latter system provides energy for numerous secondary active transport systems whilst maintaining an acidic pH within the vacuole (Nelson, 1992, Kibak, *et al*, 1992.

Vacuolar or V-ATPases are multi-subunit enzymes comprising of two parts : a peripheral, water soluble V_1 portion containing the ATP binding sites. The second subunit is the lipophilic V_o potion embedded within the membrane (Lichko and Okorokov, 1985, Anraku, *et al*, 1992, Kane and Stevens, 1992, Kibak, *et al*, 1992).

The V-ATPases are responsible for proton translocation across the tonoplast thereby maintaining the vacuolar pH. They are also responsible for the active accumulation of 10 amino acids by the vacuole. Kinetic studies have revealed the presence of 7 independant H^+/amino acid antiport systems responsible for this accumulation (Sato, *et al*, 1984, Ohsumi and Anraku, 1983).

Similarly the uptake of heavy metal cations by the vacuole is thought to be operated by a H^+/ion antiport system across the tonoplast. Evidence of the involvement of ATPase activity regarding this transport was obtained by subjecting isolated vacuoles to ATPase uncouplers and inhibitors (Lichko, *et al*, 1980, Ohsumi and Anraku, 1983, White and Gadd, 1987).

The energy capacity of the H^+ gradient can be increased by a corresponding K^+ gradient, which is an energetic partner of H^+ within fungal cells (Okorokov, *et al*, 1983, Okorokov, 1985). The efflux of vacuolar K^+ is involved in the accumulation of Mn^{2+}, Mg^{2+} and Cu^{2+} (Okorokov, *et al* 1977, Lichko, *et al*, 1980, Ohsumi, *et al*, 1988). White and Gadd (1987) demonstrated that the accumulation of Zn^{2+} within the vacuoles results in K^+ efflux.

However, an alternative school of thought has suggested that the transport of ions from the cytosol across the tonoplast is due to a specific permease. This would facilitate diffusion of ions between the cytosol and vacuole (Matile, 1978).

It is also known that the interaction of heavy metals with cellular membranes enhances their permeability, e.g. Cd^{2+} (Gadd and Mowll, 1983). As a result of such interactions the tonoplast may develop lesions, through which additional heavy metal ions may enter the vacuole and through which vacuolar ions, e.g. K^+ may be lost.

Substantial evidence indicates that on entering the vacuole the metal ions, and also amino acids, interact with polyphosphate bodies. These, the only macromolecular anions to be

found within the vacuole, serve as cation traps to form complexes involved in metabolic retention (Lichko, *et al*, 1982, Ohsumi and Anraku, 1985, Raguzzi, *et al*, 1988, Klionsky, *et al*, 1990).

In the present study the importance of vacuoles in *S. cerevisiae* as a mechanism for heavy metal accumulation was examined by investigating the uptake of heavy metal ions by isolated vacuoles.

4.2 MATERIALS AND METHODS

4.2.1 PRETREATMENT OF YEAST

The uptake of metals into the vacuole of *S. cerevisiae* is of interest. To obtain isolated vacuoles, the yeast cells were pretreated with lytic enzyme to remove the cell wall material, followed by lysis of the resultant spheroplasts which released the vacuoles as described below. The accumulation of heavy metal cations by these isolated vacuoles could then be investigated.

Washed yeast cells were pretreated following the methods of Wiemken (1975) and Rose (1988). In order to predispose the yeast cells to the action of the lytic enzyme a thiol component was employed. The thiol component, 2 mercaptoethanol acts on the disulphide bonds of the cell wall proteins, clearing them and thereby decreasing the cross-linking of the polymers.

Washed yeast cells were suspended in 10 mM sodium citrate buffer containing 0.6M sorbitol (SOB, pH 6.5) to obtain 0.2 g (wet weight) yeast/ml buffer. The solution was supplemented with 0.05 ml of 2-mercaptoethanol for every gram of the washed yeast cells. The suspension was incubated for 20 minutes at 28°C. Following incubation the cells were spun down (3 000 g x 10 min) and washed with SOB buffer to remove any residual mercaptoethanol.

4.2.2 SPHEROPLAST FORMATION

During spheroplast formation it was essential that the yeast cells remained suspended in the SOB buffer (pH 6.8). The non-metabolizing sugars and salts contained in the buffer were

required to maintain the osmotic stability of the spheroplasts. Lysing enzyme from *Cytophagia sp* containing yeast glucanase, protease and cell lytic activity was used for spheroplast formation.

The washed pretreated cells, resuspended in SOB buffer were incubated with the enzyme (1.0 mg enzyme/1.5 g wet weight yeast cells) for 2 hrs with shaking at 30°C (Wiemken, 1975).

The formation of spheroplasts was determined using phase contrast microscopy and spectroscopic studies. A few drops of the spheroplast solution were diluted in buffer (SOB, pH 6.8) or water. These suspensions were shaken occasionally for 5 minutes before reading the optical density at 600 nm. The difference between the initial and final absorptions at this wavelength for each of the solutions was calculated and compared (Rose and Veazey, 1988). Spheroplasts suspended in water were not osmotically stable compared to those suspended in buffer. This instability resulted in lysis of the spheroplasts and high differences of absorption.

4.2.3 SPHEROPLAST LYSIS AND VACUOLE FORMATION

Various methods for the lysis of spheroplasts exist, *viz.* osmotic lysis (Wiemken, 1975), metabolic lysis (Cabib and Ulane, 1973, Indge, 1968, Wiemken, 1975), mechanical disruption, ultraviolet irradiation and treatment with protein solutions (Wiemken, 1975).

Combinations of metabolic, osmotic and mechanical disruption methods together with various centrifugation techniques were employed during spheroplast lysis and vacuole isolation. Similar to Cabib (Cabib and Ulane, 1973) the liberation of vacuoles from spheroplasts by osmotic lysis during this study was very poor. Metabolic lysis was therefore used as a method to obtain vacuoles from spheroplasts.

The isolation of the vacuoles was obtained following the method of Indge (1968), whereby the yeast spheroplasts were metabolically lysed to release the intact vacuoles.

One volume of the spheroplast-SOB suspension was added to 50 volumes of 10 mM Imidazole-HCl buffer (pH 6.5) containing mannitol (10% w/v) and 5 mM EDTA. After

equilibration of the solution at room temperature for 10 minutes, after which a 10% w/v glucose solution (2.5 ml glucose/1 ml spheroplasts) was added to start the reaction. The mixture was incubated at 30°C for 30 minutes with gentle shaking.

The released vacuoles were concentrated into a pellet by centrifuging at 2 000 g for 4 minutes. To obtain the vacuolar fraction this pellet was suspended in Imidazole-HCl buffer containing 10 mM mannitol (0.1 ml vacuole suspension per 1 ml buffer). The gelatinous material containing the cell debris readily sedimented out through gravitational forces, leaving a vacuole rich supernatant which was easily removed with a pasteur pipette.

4.2.4 VACUOLE INTEGRITY

To determine the integrity of the vacuoles the vacuolar fraction was stained using neutral red dye (Peterson, 1979). The staining proceeded for one hour in freshly prepared neutral red dye made up to 0.01% in 0.8M phosphate buffer (pH 7.2). To determine the vacuole integrity the stained fraction was rinsed, mounted in buffer on slides and viewed microscopically. Intact vacuoles stained brick red to red in colour. This was due to the ionic form of the dye accumulating therein via an ion trap mechanism.

4.2.5 METAL UPTAKE

To determine metal uptake by vacuoles, 1 ml of the vacuole solution was incubated with 1 ml of the metal ion solution. The chloride salts of Cd^{2+}, Co^{2+} and Cu^{2+} were used. The isolated vacuoles were exposes to concentrations of these metals ranging from 5 - 60 μM over a time period of 120 minutes whilst being kept on ice. After incubation the vacuole-metal solution was filtered through 0.45 μM pore size, 25 mm diameter membrane filters (Millipore) according to the method of Norris and Kelly (1977).

The vacuole fraction remaining behind on the filters was washed with 1 ml of Imidazole-HCl buffer containing 5 mM EDTA. The filtrate (3 ml) contained metal ions not taken up by the vacuoles as well as those washed from the exterior of the vacuole surface.

The filters, containing the vacuole-metal fraction, were placed in 10 ml of 2M HCl and digested (100°C, 2 hrs) to release the accumulated metal ions.

Levels of accumulated and free metal ions were detected using atomic absorption spectroscopy (Varian AA - 1275 Spectrophotometer) at the relevant wavelengths for each of the respective metals (Cd: 228.5 nm, Co: 240.7 nm, Cu: 324.8 nm).

4.2.6 ATPase INHIBITION

To determine the role of the V ATPase H^+/antiport channel system in the uptake of divalent metal cations into the vacuole, an ATPase uncoupling agent was employed.

Prior to addition of the 1 ml of metal solution, 200 μl of a 2 mM solution of the ATPase uncoupling agent 2,4 Dinitrophenol (DNP) was added. Incubation of the vacuole-DNP fraction on ice for 10 minutes enabled the DNP to interact with the vacuolar ATPase. The appropriate metal solution (1 ml) was added resulting in the final concentrations of 182 μM DNP in solution. The remainder of the procedure followed the abovementioned methodology (Section 4.2.5).

4.3 RESULTS

Isolated yeast vacuoles were found to be capable of accumulating metal cations. When exposed to varying external concentrations of the divalent cations (5 - 60 μM), the vacuole suspensions accumulated these ions in the relative affinities of $Cu^{2+} > Co^{2+} > Cd^{2+}$.

The accumulation of the three metal cation species within the vacuole showed similar trends (Figures 4.1, 4.2 and 4.3). The majority of the heavy metal cations were accumulated by the vacuole within the first 30 minutes of exposure to the test metals. This initial rapid uptake phase was followed by a secondary phase. During this later phase, the levels of metal cations within the vacuoles either gradually increased e.g. Cu^{2+} and Co^{2+}, or alternatively levelled out, *viz*, Cd^{2+}. Maximum uptake depended on the metal species present. A profile of the atomic radii of these three metals revealed an inverse relationship between uptake and size (Figure 4.4).

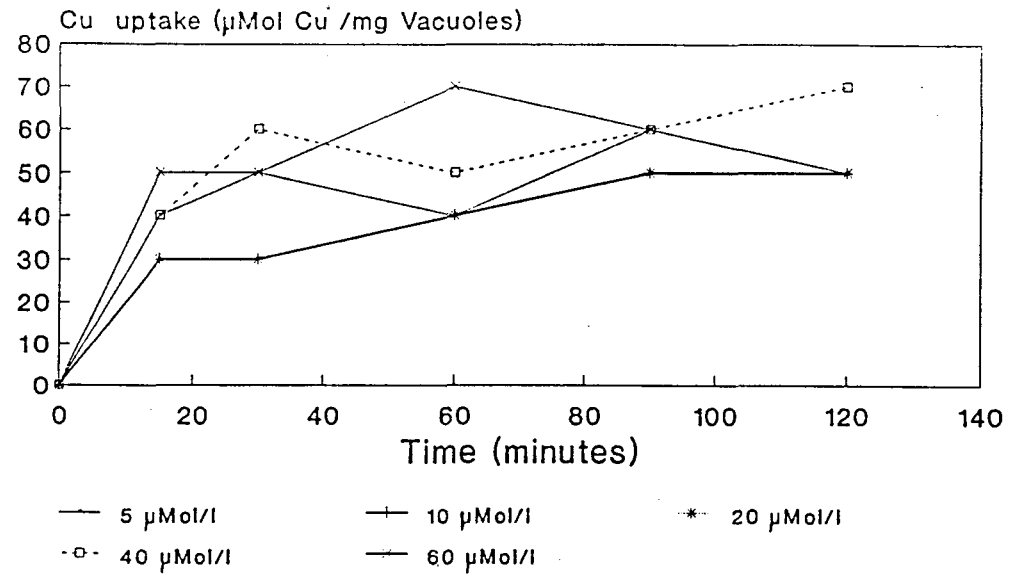


Figure 4.1: Cu^{2+} uptake over time by yeast vacuoles exposed to varying external concentrations of Cu^{2+}

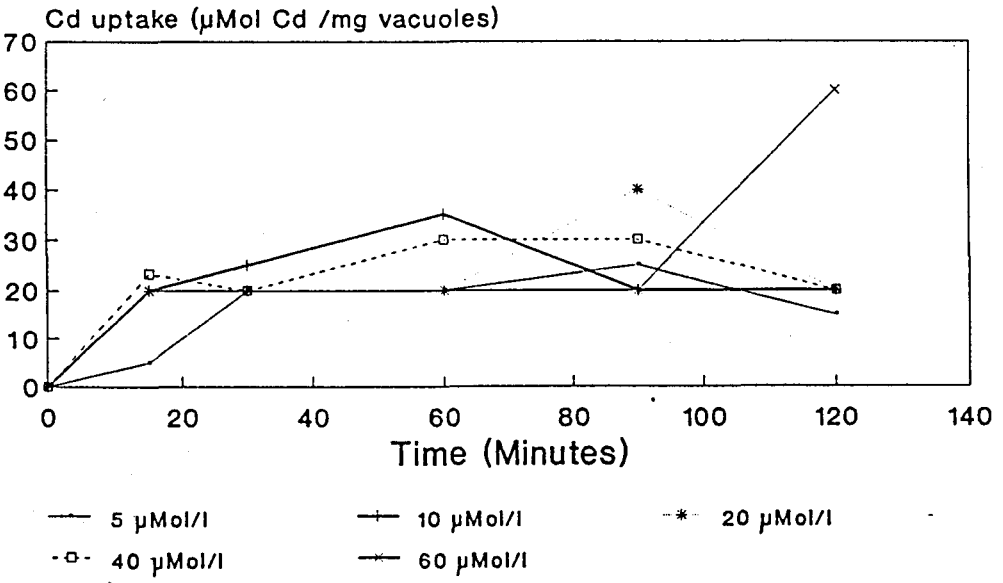


Figure 4.2: Cd^{2+} uptake over time by yeast vacuoles exposed to varying external concentrations of Cd^{2+}

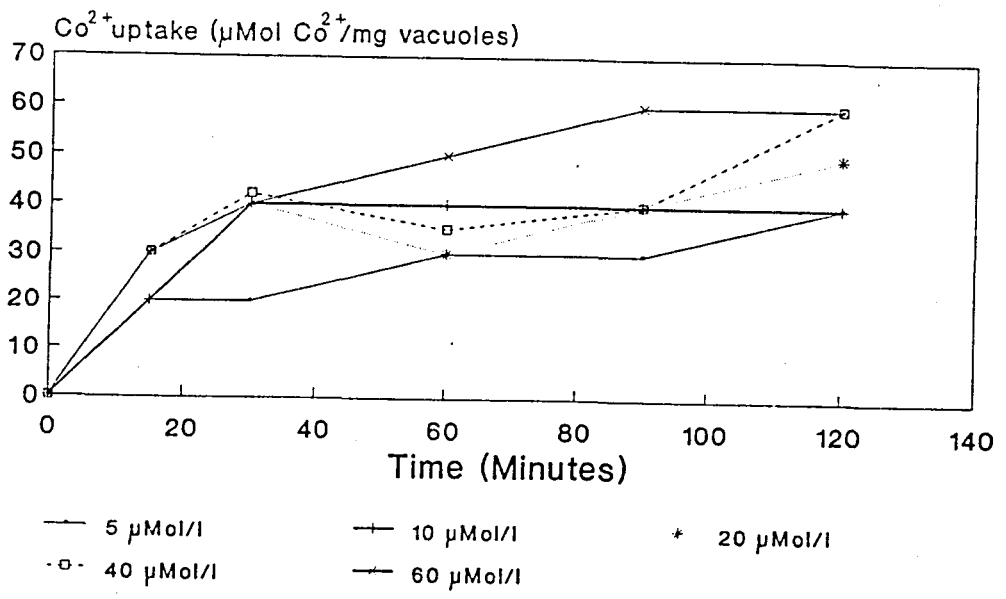


Figure 4.3: Co^{2+} uptake over time by yeast vacuoles exposed to varying external concentrations of Co^{2+}.

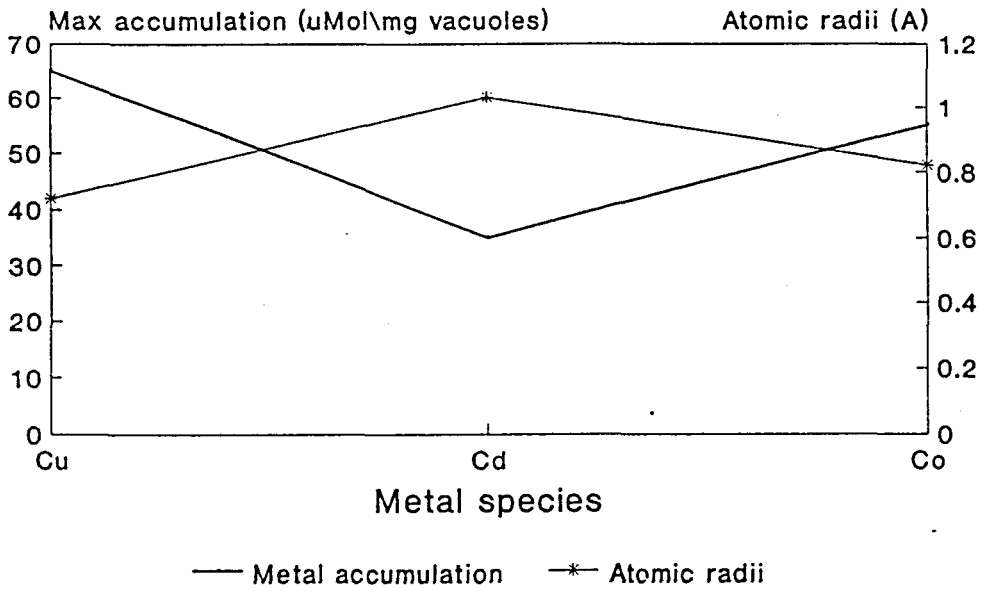


Figure 4.4: The inverse relationship between uptake of metal ions into vacuoles and their atomic radii

The ATPase uncoupler, 2,4 Dinitrophenol (DNP) did not have a strong inhibitory effect on the uptake of Co^{2+} and Cd^{2+} into the vacuoles (Figures 4.5 and 4.6). The accumulation of Cu^{2+} after 120 minutes by DNP treated vacuoles was less than in untreated vacuoles (Figure 4.7). However, the metal accumulation of cations (0 - 15 minutes) within the DNP pretreated vacuoles was comparable to those of the untreated vacuoles irrespective of the metal cation species or their external concentrations. This suggests that the metal cations entered into the vacuoles by alternative routes.

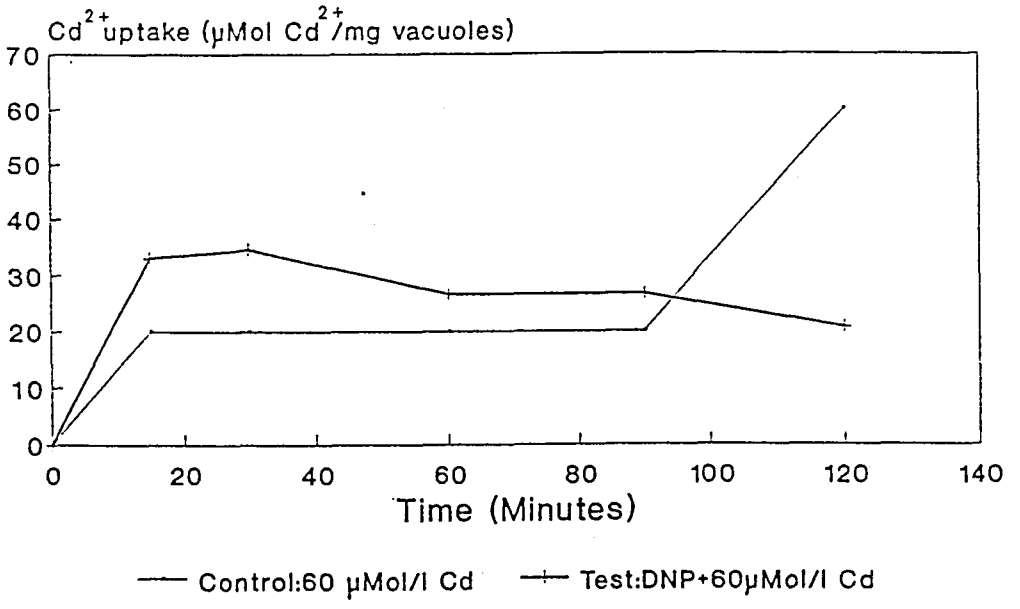


Figure 4.5: The effect of DNP on the uptake of Cd^{2+} into the yeast vacuole

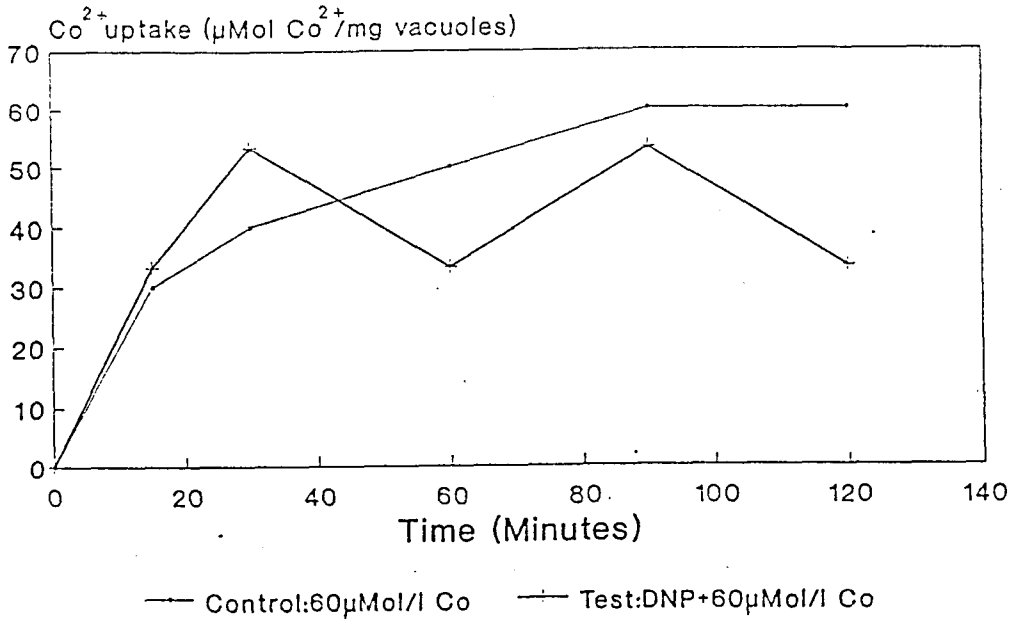


Figure 4.6: The effect of DNP on the uptake of Co^{2+} into the yeast vacuoles

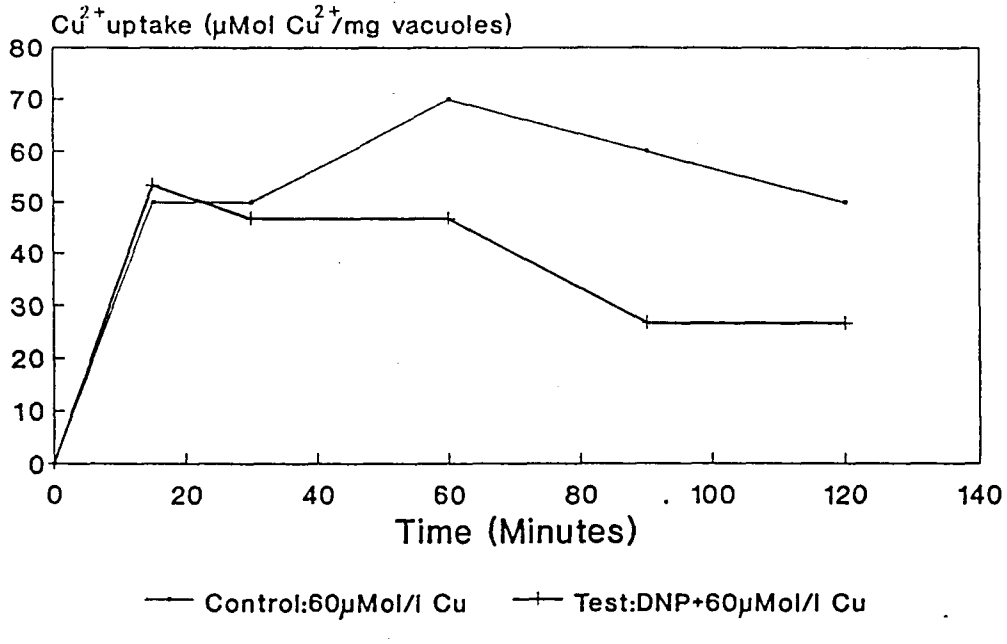


Figure 4.7: The inhibition of Cu^{2+} uptake into the yeast vacuole by DNP

4.4 DISCUSSION

Uptake of heavy metal cations from solution by isolated vacuoles prssented evidence of a mechanism different from that of the cell wall binding and intracellular accumulation described in Chapters 2 and 3. Whilst inhibiting vacuolar Cu^{2+}, DNP, an ATPase uncoupling agent, failed to inhibit the uptake of Co^{2+} and Cd^{2+} into the vacuole. This suggests that the latter gained entry into the vacuole via an alternative pathway(s). Cu^{2+}, although affecting the permeability of the plasma membrane, does not affect the permeability of the tonoplast (Ohsumi, *et al*, 1988). On addition of DNP, entry of Cu^{2+} into the vacuole was therefore limited. Entry of Co^{2+} and Cd^{2+} into the vacuole was probably facilitated through lesions in the tonoplast.

Deposition of metal cations within vacuoles occurred over time. Due to the vacuole occupying a large portion of the cell (Boller and Wiemken, 1986, Jones and Gadd, 1990), a high amount of metal cations were sequestered within this organelle. Once within the vacuole, the positively charged ions associated with the negative polyphosphate bodies, which could explain the large phosphorous peak present in the EDAX spectra of all cells (Chapter 3).

CHAPTER 5

IMMOBILIZATION OF YEAST FOR BIOACCUMULATION OF METAL CATIONS

5.1 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 entrapment 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), alginate (Flink and Johansen, 1985) 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).

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).

An alternative system of cell immobilisation to the calcium and gel matrix system described above is to use microfiltration systems such as tangential membrane filtration. 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 5.1). 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.

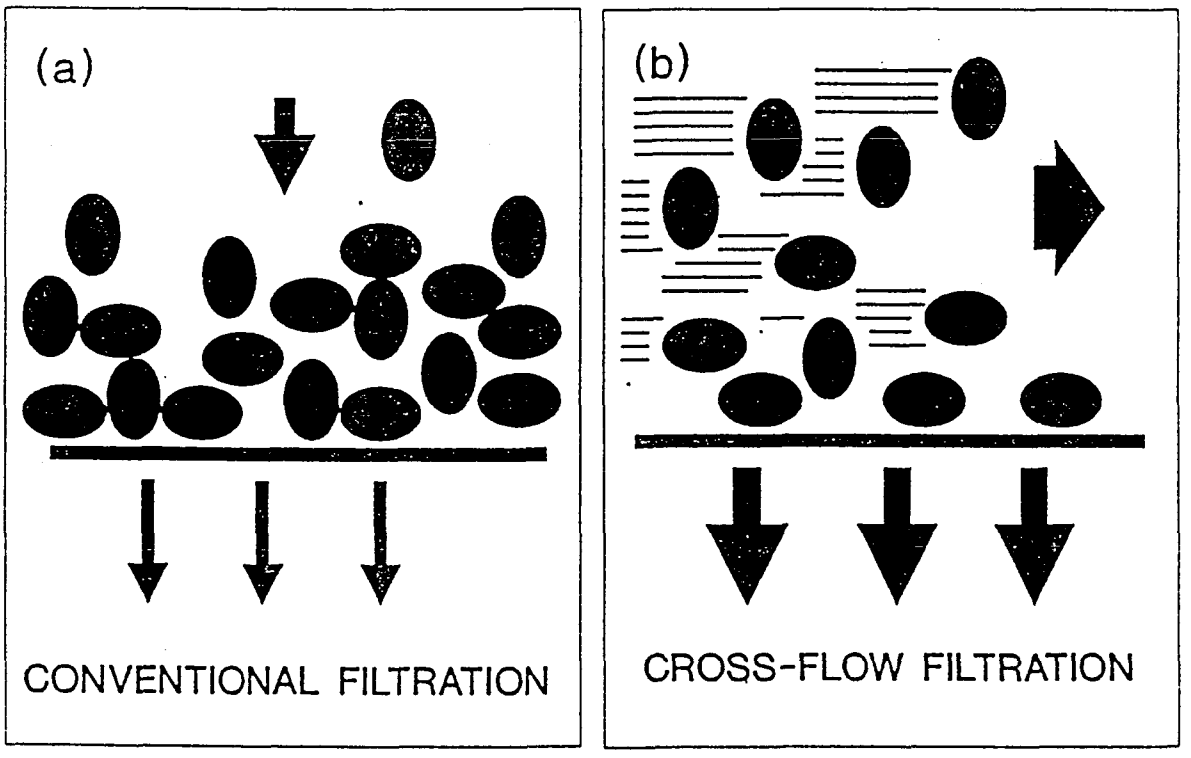


Figure 5.1: Schematic representation of conventional (a) and tangential flow (b) filtration processes

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 macro-molecules and colloids. Cross-flow microfiltration (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 biofouling 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 graident (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 mocroporus film (Lopez-Leiva and Gekas, 1986).

This aim of this section of the study was to examine the potential of two different immobilisation systems, a gel matrix column system and microfiltration systems, for entrapment of yeast cells for use in heavy metal bioaccumulation studies.

5.2 MATERIALS AND METHODS

5.2.1 COLUMN

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 ml physiological saline (0.15 mol/ℓ NaCl) at 8°C.

Monomer: Acrylamide monomer (7.5 g) plus *N,N'*-methylene-bisacrylamide (0.4 g) was dissolved in 24 ml 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 ml of 2.5% TEMED (*N,N,N'N'*-tetramethylethylene-diamine), a catalyst for free radical propagation during the polymerization process, and 5 ml 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.

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.

Experimental Conditions for Column System

Flow rate: 1 ml/min. Fraction volume: 10 ml. Column height: 10 cm. Column volume: 20 ml. Temperature: 20°C $\pm$ 2°C.
Metal stock solutions:

(a) 200 μmol/ℓ solution of metal chloride in 5 nmol/ℓ HEPES buffer pH 7.2 (Highveld Biological Ltd).

(b) 200 μmol/ℓ 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 ml of 1.0 mmol/ℓ HEPES buffer pH 7.2. Buffer (30 ml) 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.

5.2.2 ULTRAFILTRATION SYSTEM

The use of ultrafiltration was investigated to determine its potential for harvesting yeast cells after bioaccumulation. The equipment used is shown schematically in Figure 5.2.

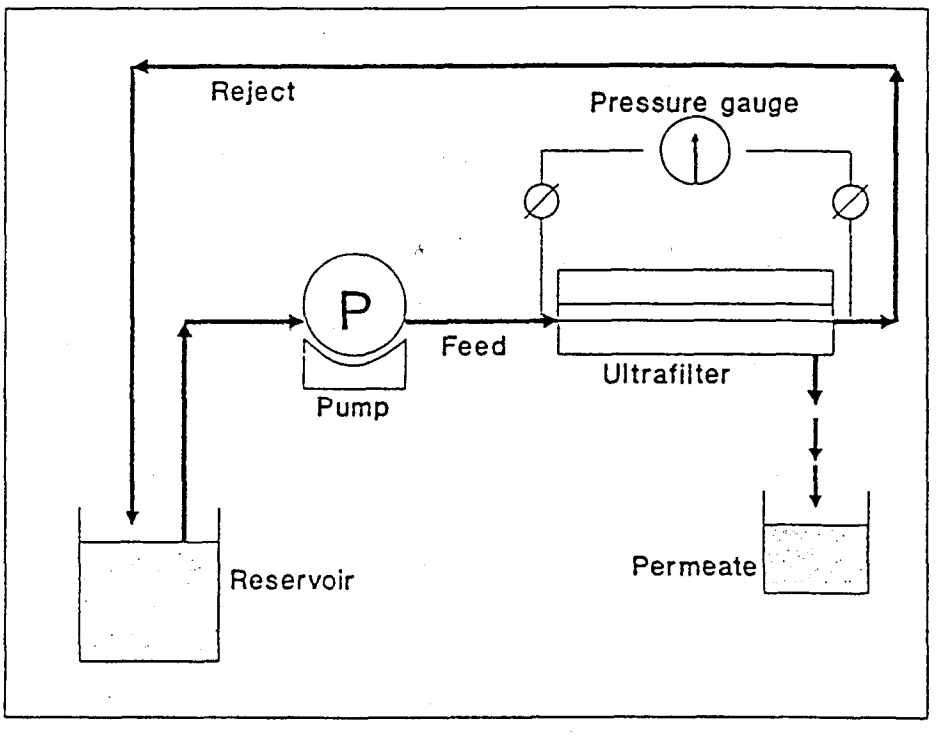


Figure 5.2: Cross-flow ultrafiltration system. Yeast cell suspensions are pumped under pressure into the lumen of the hollow fibres within the hollow fibre cartridge, and the resultant permeate drains to the exterior where it is collected.

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 ℓ/hr
Membrane area: 9.4×10^{-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 ℓ ultra-pure water and harvested according to the experimental conditions stated above.

Determination of Initial Flux Rates

One litre of 5 mmol/ℓ HEPEs buffer pH 7.2 containing 200 μmol/ℓ $CuCl_2$ 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.

5.2.3 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.

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 as depicted in Figure 5.3. Unlike the ultrafiltration experiments the yeast cell suspensions were circulated around the exterior of the hollow fibres and the permeate passed into the lumen.

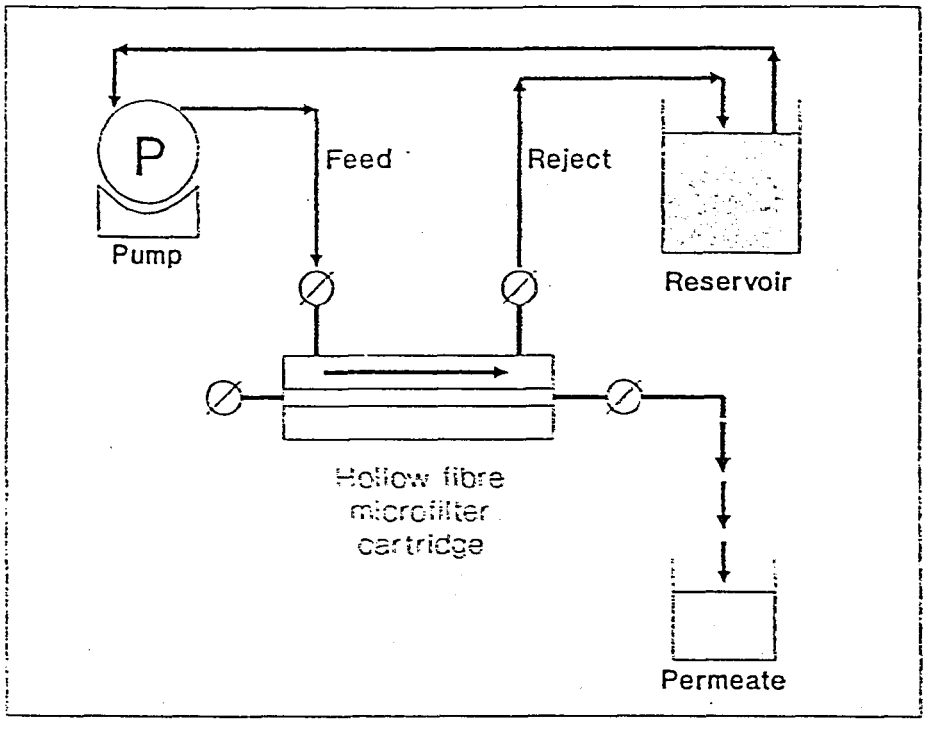


Figure 5.3: Microfiltration equipment set-up (schematic). There were 7 hollow fibres within the cartridge with a total surface area of 0.013 m^2.

(a) Serial microfiltration for bioaccumulation of copper

S. cerevisiae cells (1.4 g wet mass) were suspended in a 1 ℓ solution of 200 μmol/ℓ $CuCl_2$ buffered with 5 mmol/ℓ 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 μmol/ℓ $CuCl_2$) maintained the volume in the influent reservoir at its initial level while permeate was collected in separate 1 ℓ 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 ℓ 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.

(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 μmol/ℓ) was added to the reservoir in such a manner as to maintain a constant volume in the reservoir during the period fo 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 bioaccumualte 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 μmol/ℓ. 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 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.

5.3 RESULTS

5.3.1 COLUMN IMMOBILISATION SYSTEM

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 5.4 to 5.6 and summarised in Table 5.1.

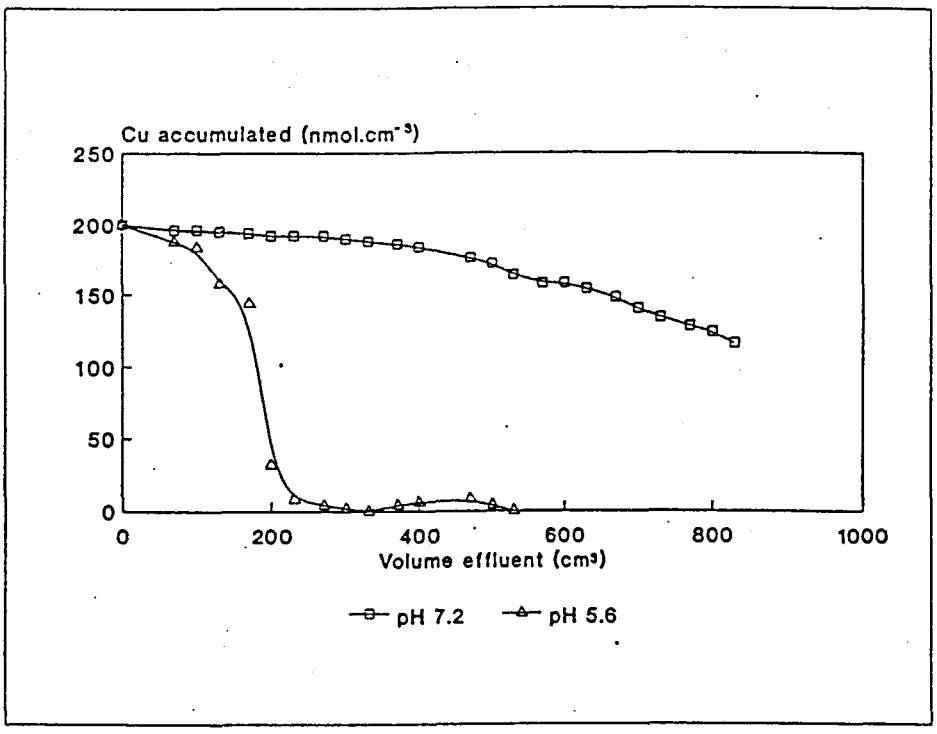


Figure 5.4: Copper accumulation by a column packed with polyacrylamide immobilized yeast cells. Influent pHs were 7.2 and 5.6

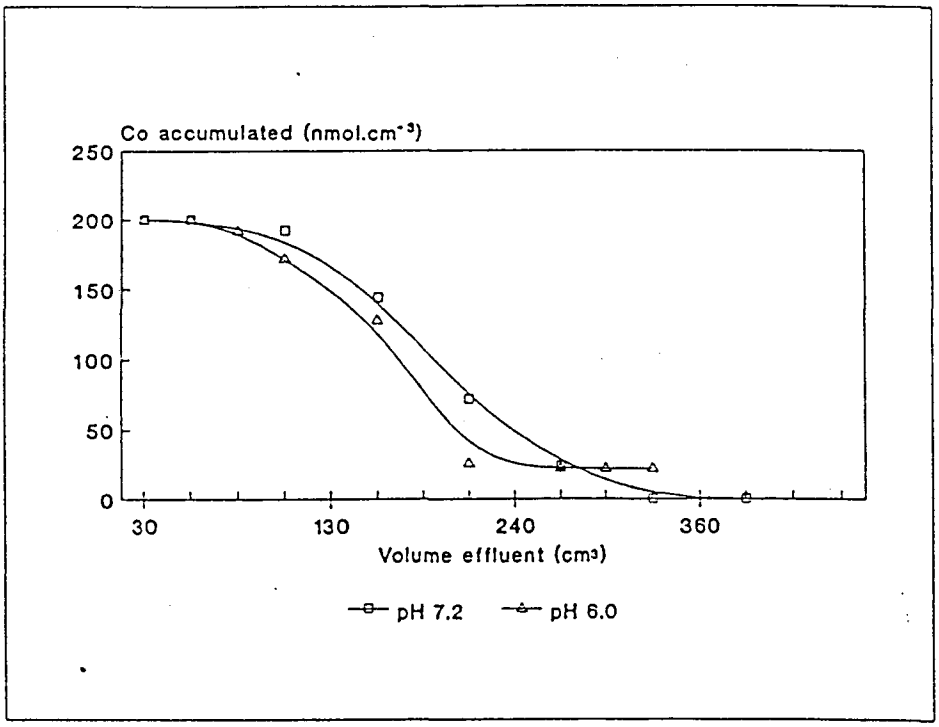


Figure 5.5: Cobalt accumulation by a column packed with polyacrylamide immobilized yeast cells. Influent pHs were 7.2 and 6.0.

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.

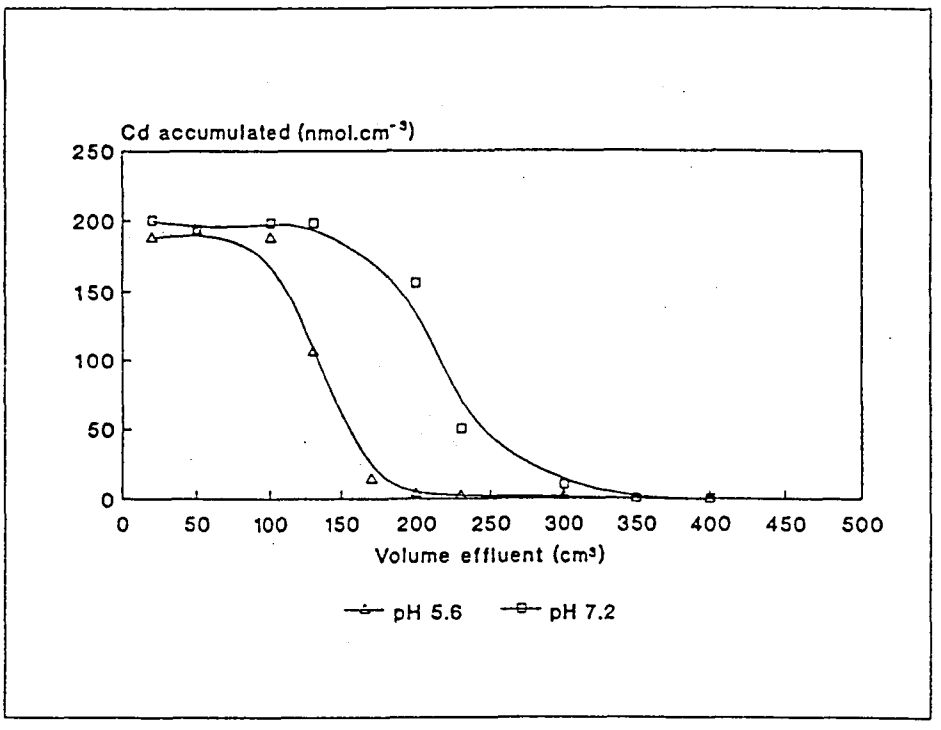


Figure 5.6: Cadmium accumulation by a column packed with polyacrylamide immobilized yeast cells. Influent pHs were 7.2 and 5.6.

Table 5.1: 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 5.7 to 5.9). 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 5.10). During copper accumulation in the presence of calcium the effluent calcium concentration exceeded the influent clacium, suggesting calcium loss may be related to the accumulation process in some way. Later experiments showed that calcium release during cadmium accumulation appears to be very closely related to the ability of the biomass to accumulate cadmium at pH 7.2 (Figure 5.11) and 5.6 (data not shown).

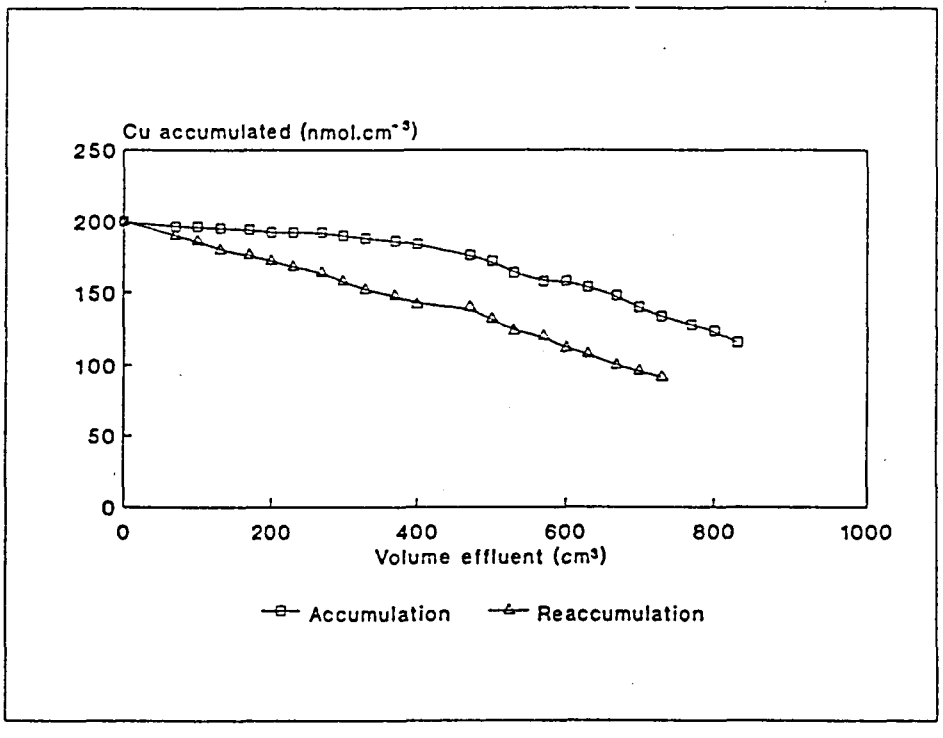


Figure 5.7: Copper accumulation and re-accumulation by a column of immobilized yeast cells. After accumulation the biomass bound metal was eluted with an EDTA solution and washed with ultra-pure water. This was followed by a second accumulation cycle.

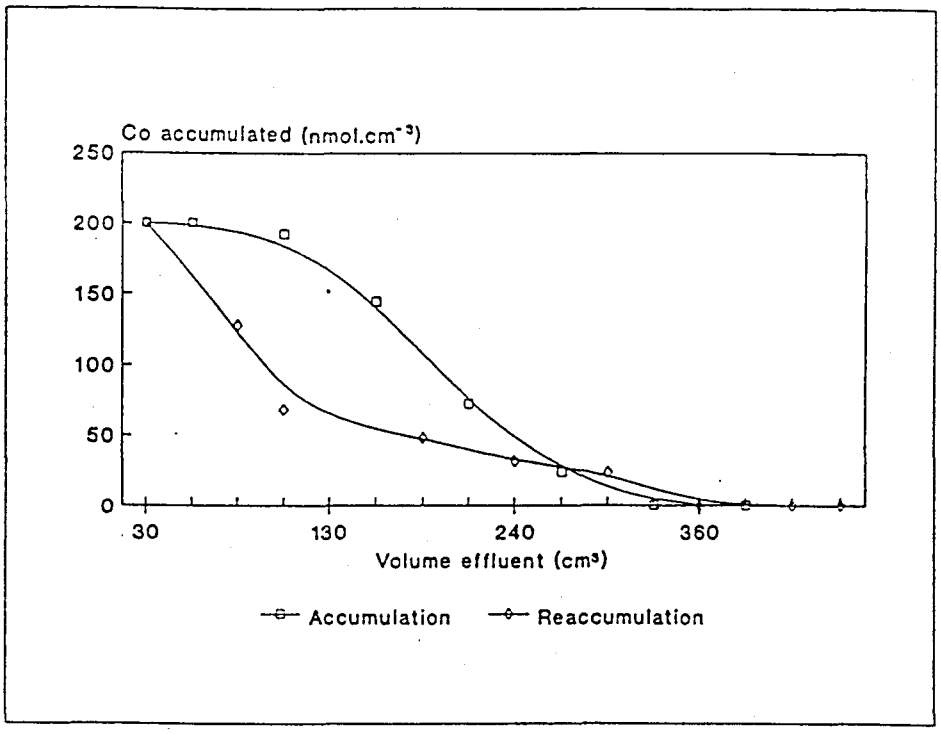


Figure 5.8: Cobalt accumulation and re-accumulation by a column of immobilized yeast cells. After accumulation the biomass-bound metal was eluted with an EDTA solution and washed with ultra-pure water. This was followed by a second accumualtion cycle.

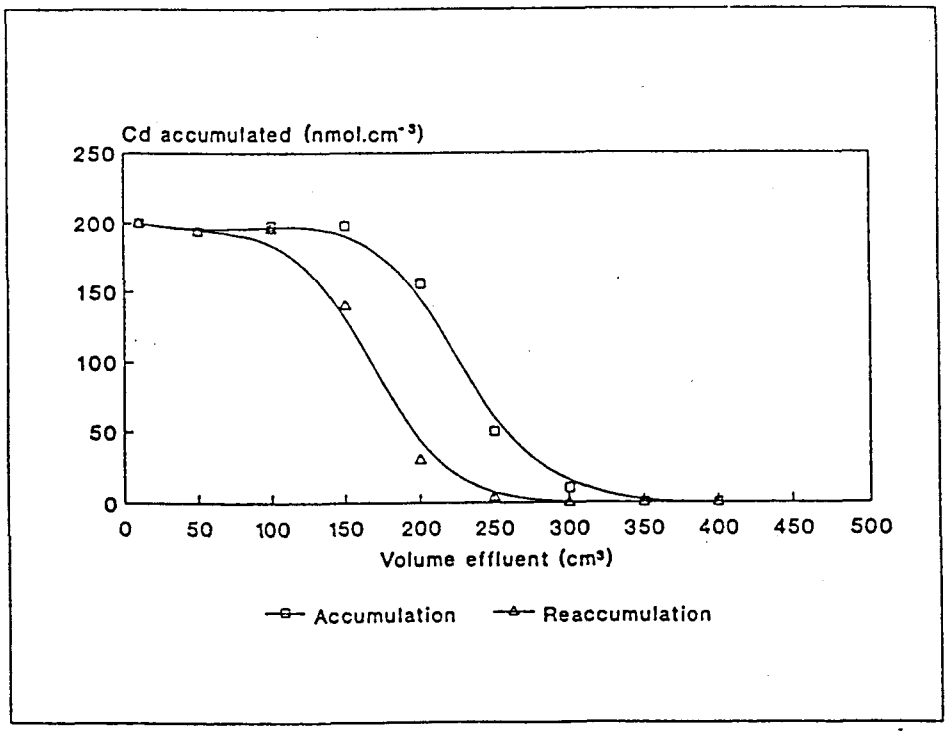


Figure 5.9: Cadmium accumulation and re-accumulation by a column of immobilized yeast cells. After accumulation the biomass-bound metal was eluted with an EDTA solution and washed with ultra-pure water. This was followed by a second accumulation cycle.

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 efficiency is dependent on the ambient copper concentration. As can be seen in Figure 5.12 a 1 mmol/ℓ 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 polyacrylamide gel may be responsible for this effect.

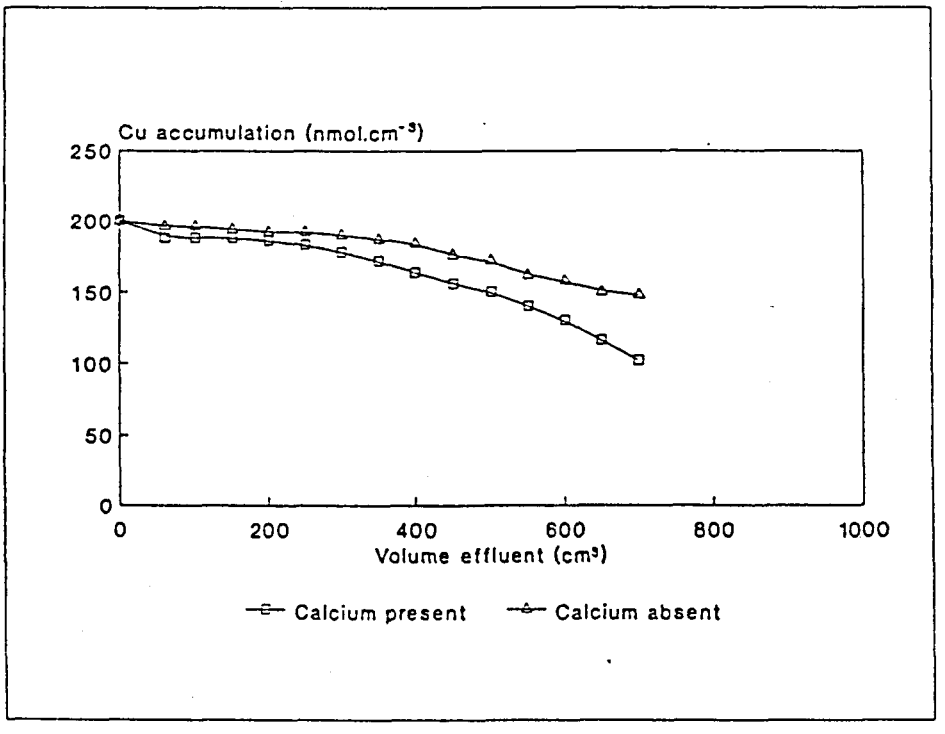


Figure 5.10: Copper accumulation during calcium competition by a column packed with polyacrylamide immobilized yeast. Influent pH was 7.5.

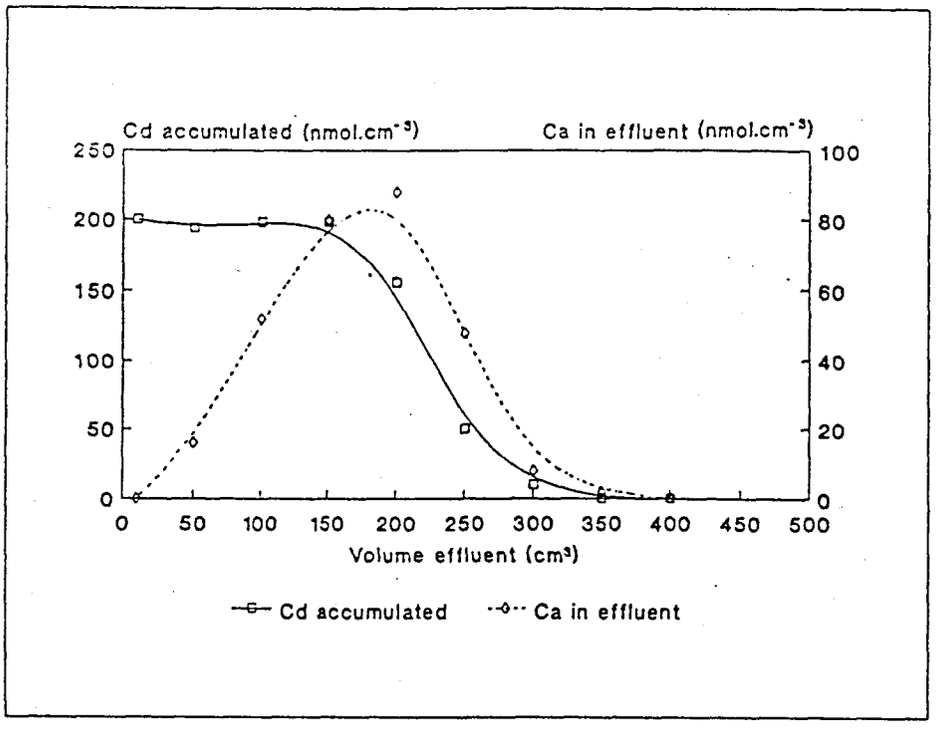


Figure 5.11: Calcium release from a column packed with polyacrylamide immobilized yeast cells during acccumulation of cadmium. Influent pH was 7.2.

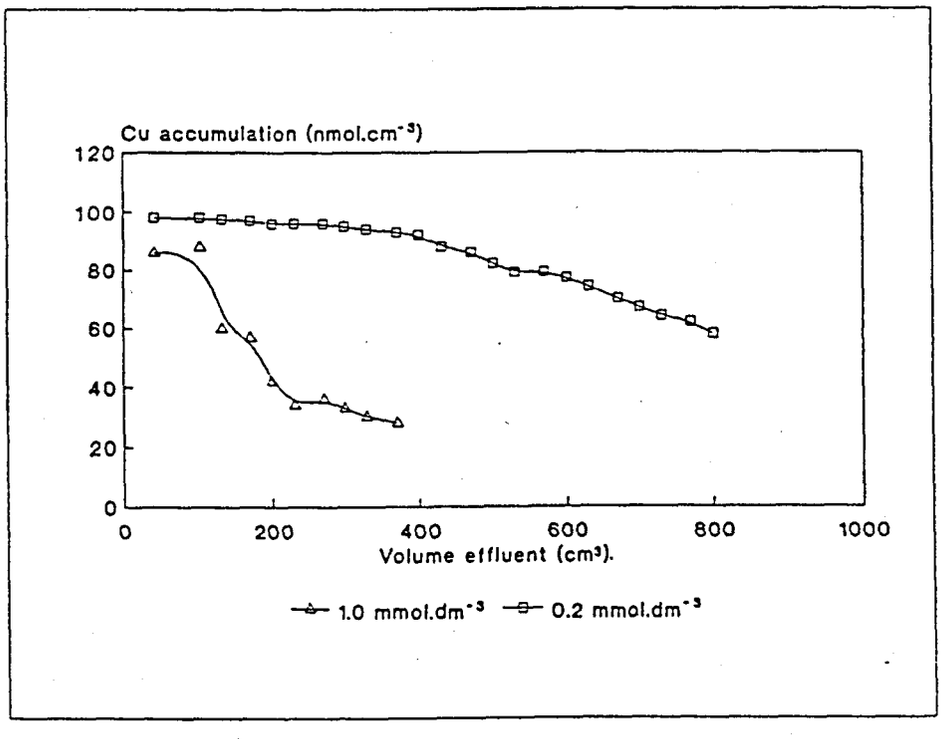


Figure 5.12: Copper bioaccumulation at two influent copper concentrations (1.0 mmol/ℓ and 0.2 mmol/ℓ) by a column of polyacrylamide immobilized yeast cells.

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.

5.3.2 ULTRAFILTRATION SYSTEM

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 5.13). Moreover, the initial flux rate of native cells and cells which had accumulated copper were similar (results not shown).

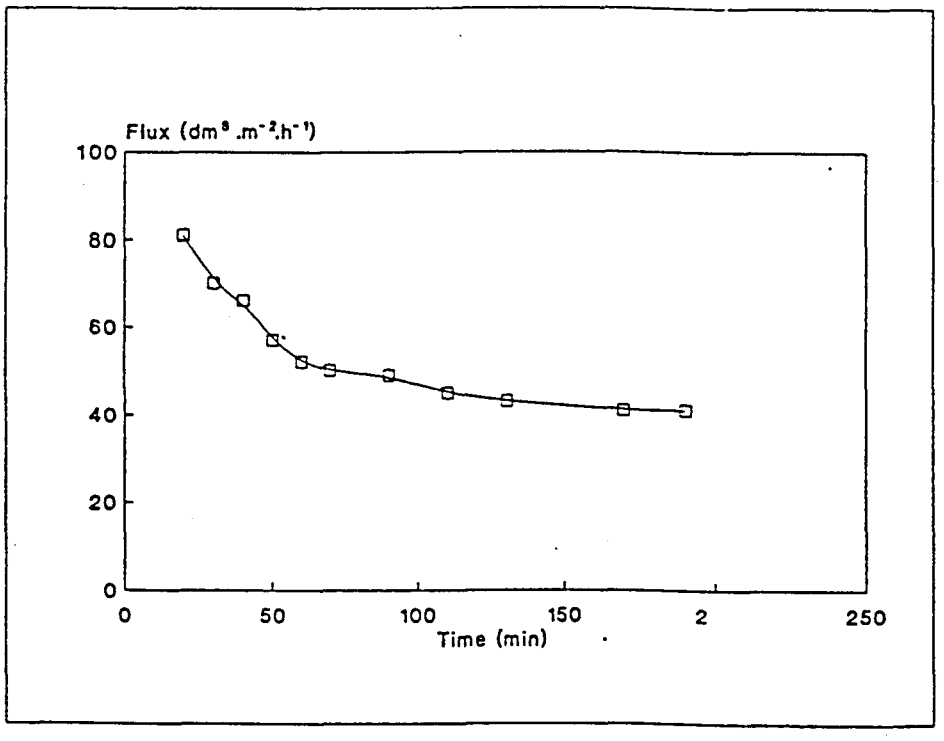


Figure 5.13: The flux rate of yeast cell ultrafiltration using a polysulphone hollow fibre cartridge.

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 beencaused 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.

5.3.3 CROSS-FLOW MICROFILTRATION SYSTEMS

The use of sequential heavy metal accumulation by biomass was demonstrated to be effective (Figure 5.14), but the intracellular cation release by the cells accumulates downstream in the process (Figures 5.15 to 5.17) and could result in cation competition for binding sites in later cycles.

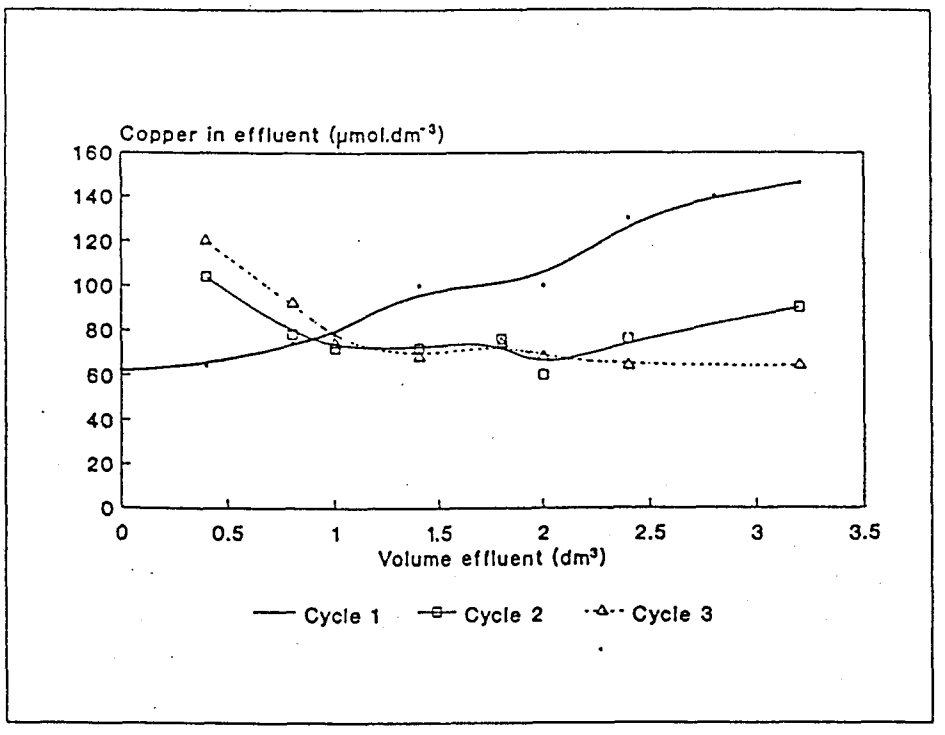


Figure 5.14: Copper accumulation by application of serial cross-flow microfiltration. Reject: 0 ℓ/ℓ; feed: 0.2 ℓ/min Flux rate: 9.2 x 10^2 ℓ/m/hr.

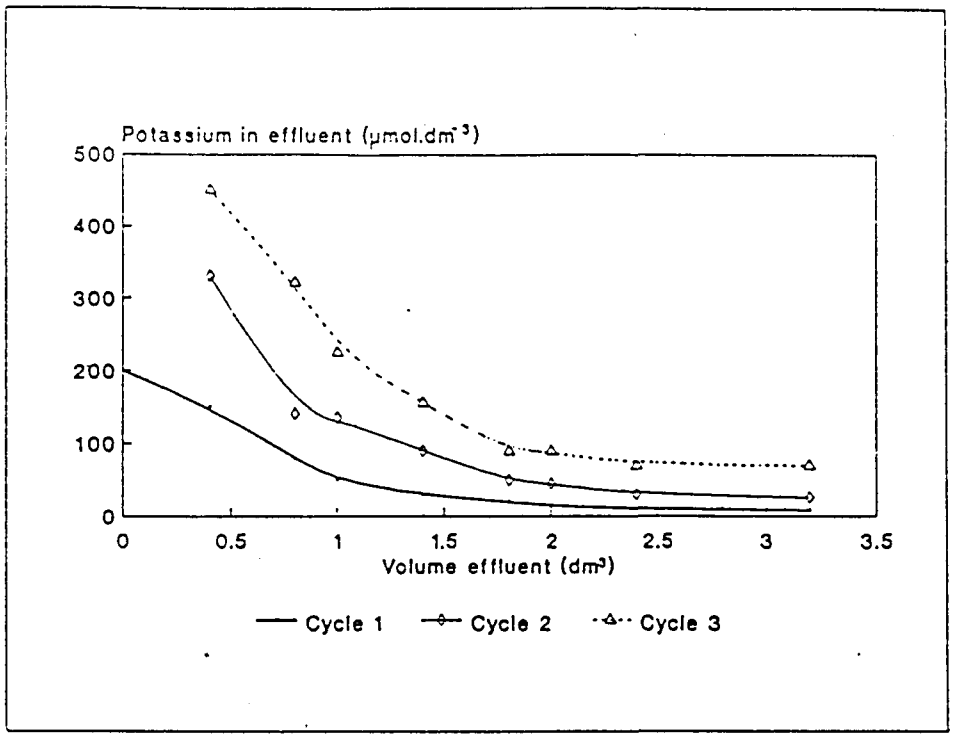


Figure 5.15: Potassium release during copper accumulation by application of serial cross-flow microfiltration.

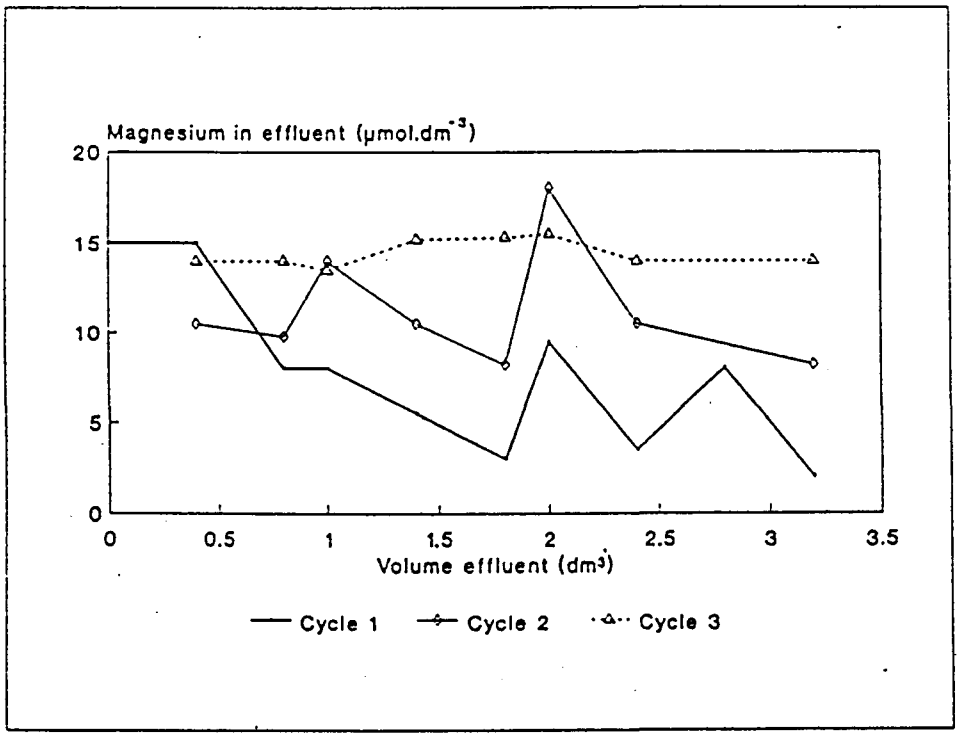


Figure 5.16: Magnesium release during copper accumulation by application of serial cross-flow microfiltration

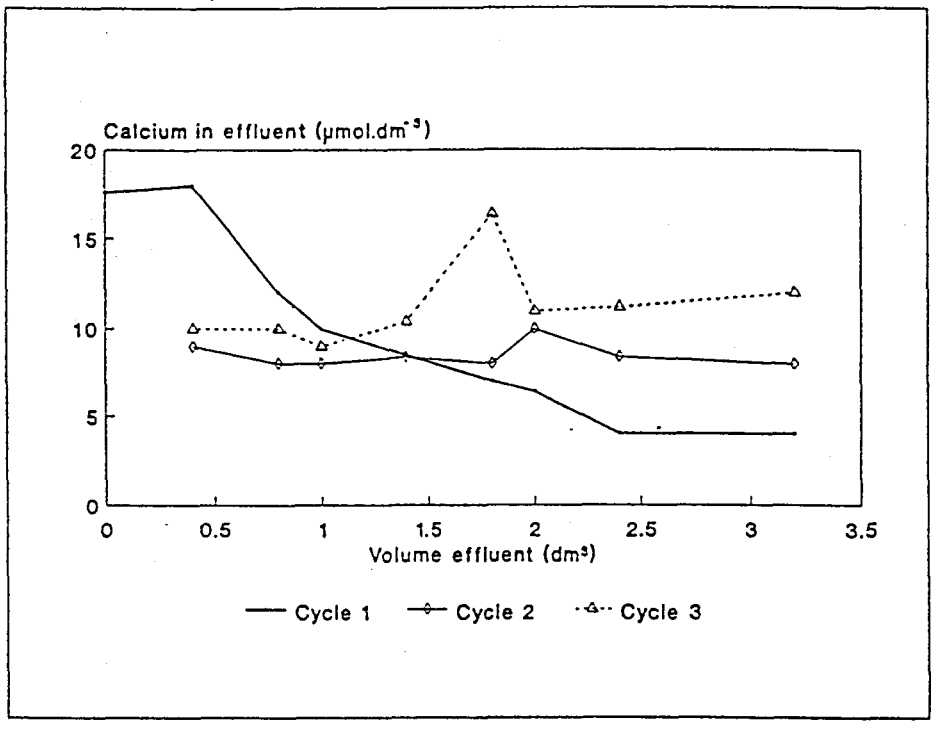


Figure 5.17: Calcium release during copper accumulation by application of serial cross-flow microfiltration.

Harvesting of yeast biomass during copper accumulation (Figure 5.18) and an alternative process of pre-packing of microfiltration filter cartridges with yeast prior to accumulation of copper (Figure 5.19) were compared (Figure 5.20). In each case substantial quantities of copper were accumulated.

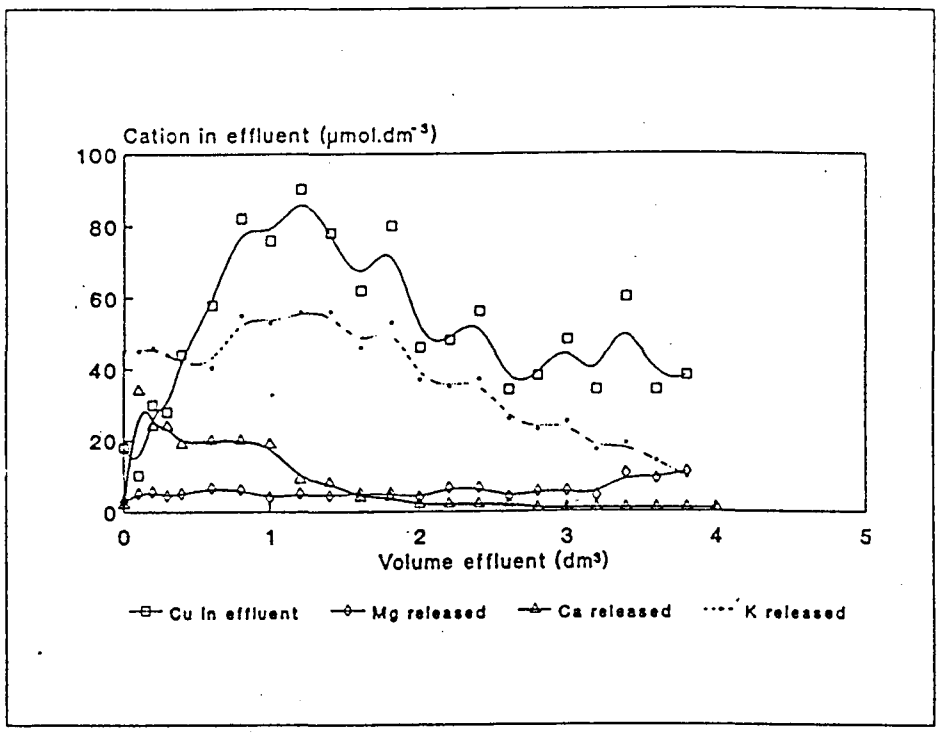


Figure 5.18: Harvesting of yeast by CFMF during bioaccumulation of copper. Temperature: 30°C, flux rate: 9.2 x 10^2 $\ell/m^2/hr$

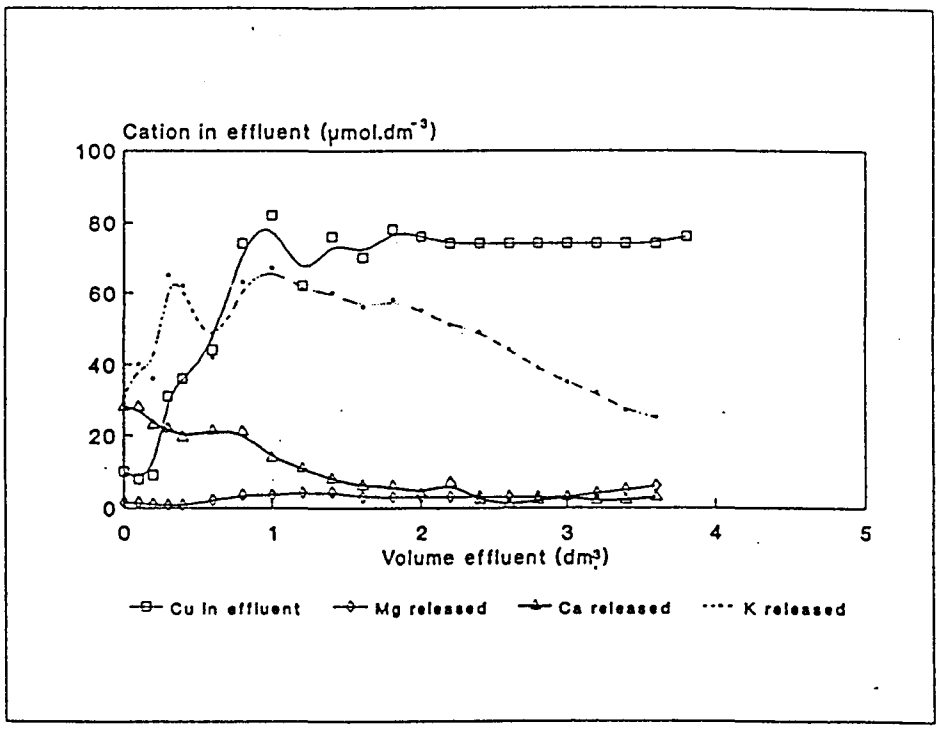


Figure 5.19: Sequential packing of the hollow fibre cartridge with yeast, followed by bioaccumulation. Temperature: 30°C, flux rate: 12.5 x 10^2 $\ell/m^2/hr$

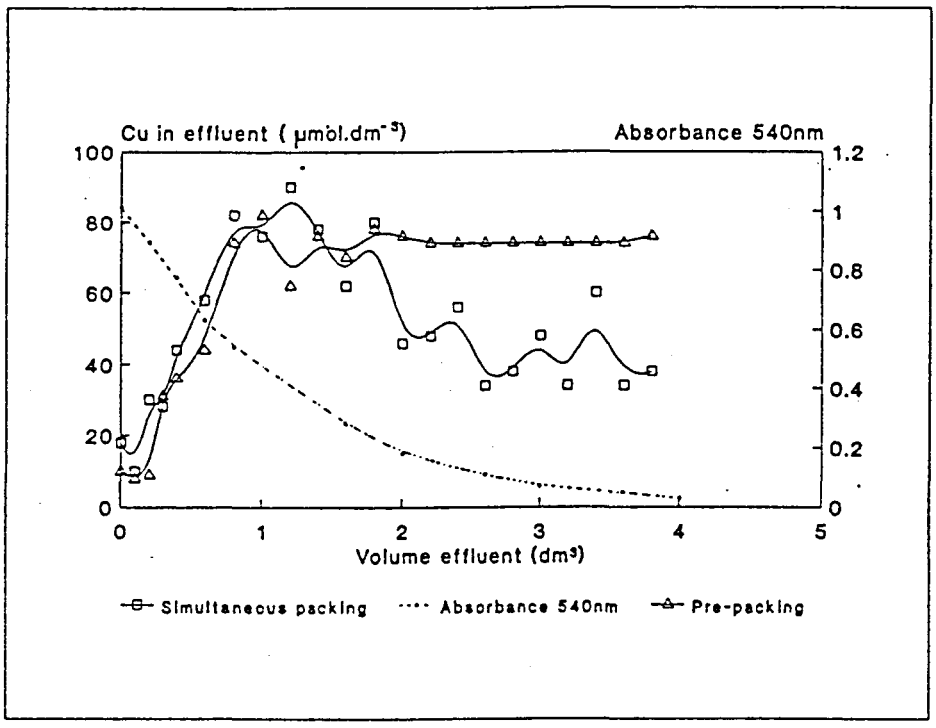


Figure 5.20: Comparison of copper bioaccumulation when either pre-packing the CFMF filter with yeast biomass prior to accumulation or simultaneous accumulation and harvesting. Yeast cell packing onto the CFMF filters during simultaneous harvesting/accumulation is represented by the decreasing absorbance (at 540 nm) of the solution in the reservoir.

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 5.21). The metals Cr^{3+}, Cu^{2+} and Pb^{2+} were accumulated in large quantities, while Cd^{2+} was accumulated to a much lesser extent.

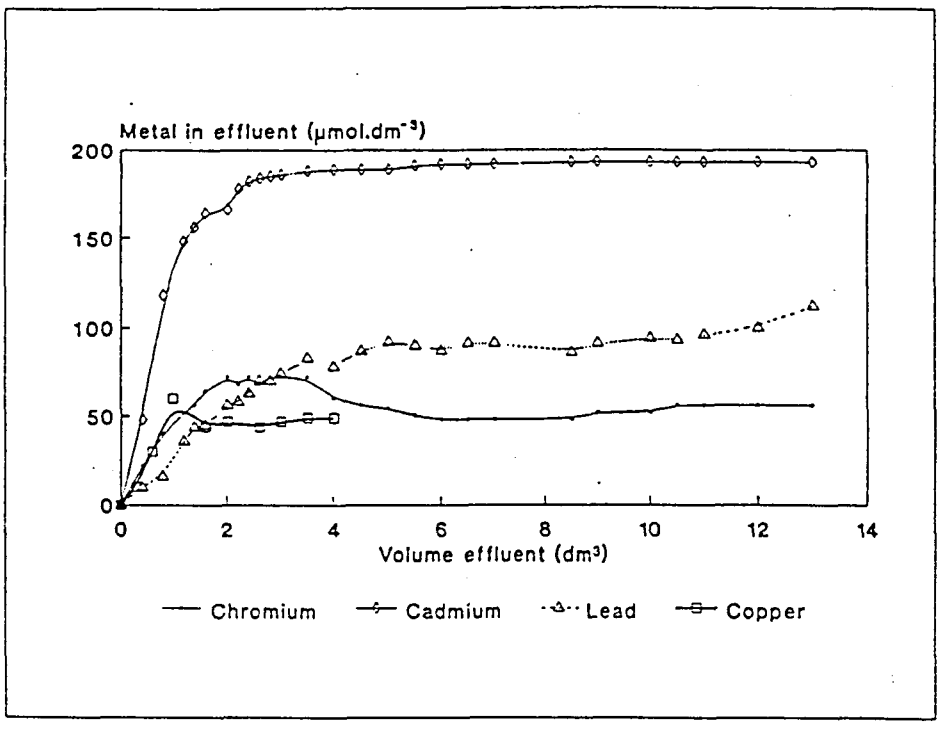


Figure 5.21: Bioaccumulation of metal cations from domestic tapwater artificially contaminated by metal chloride salts. Cu^{2+} accumulation was only monitored for the first 4 ℓ.

The system was also capable of haze removal from lead contaminated tapwater possible reflecting the retention of colloids by the system.

The variation of flux rate with addition of tapwater to a cartridge packed with yeast cells was determined (Figure 5.22). The flux rate increased with passage of permeate across the membrane.

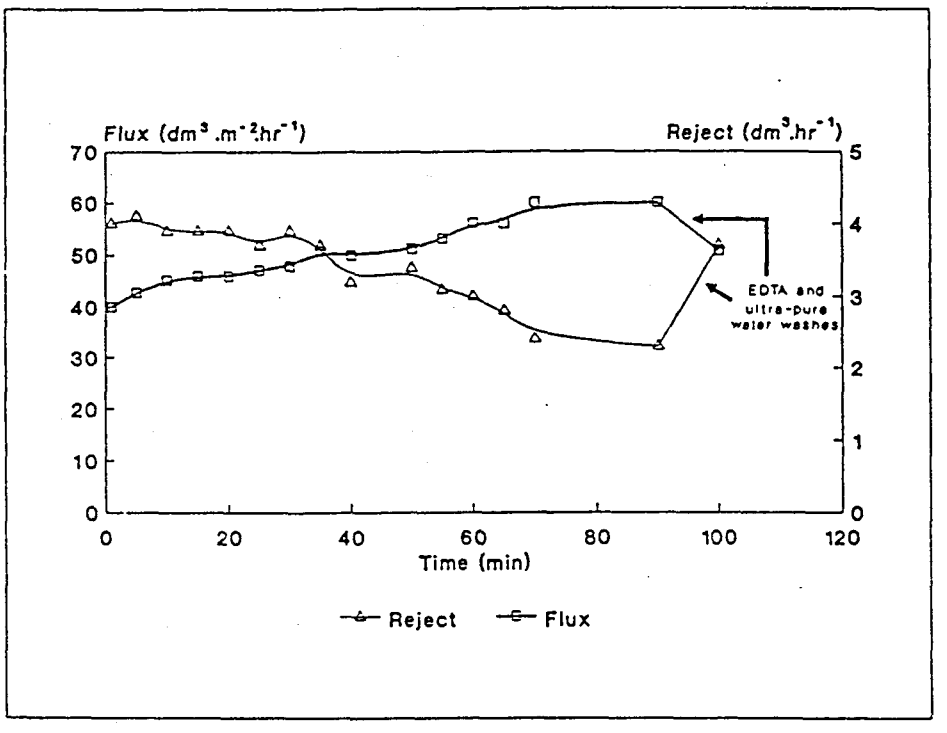


Figure 5.22: The flux rate of hollow fibre cross-flow microfiltration during yeast biomass harvesting as a function of time, and the effect of addition of the chelating agent EDTA.

5.4 DISCUSSION

5.4.1 COLUMN IMMOBILIZED SYSTEM

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 column immobilized 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 system 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 on column immobilization that metal uptake is dependent on metal concentration (Brady and Duncan, 1993a,b). However, if the column experiments are examined > 99% removal of metal from solution is found. 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 5.1). As the liquid leaves the first theoretical plate it encounters a second plate, and perhaps 55% of the remaining metal is retained. The results of a number of repetitions of this process can be seen in Table 5.2.

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 5.2: Metal cation accumulation by a theoretical bioaccumulation column

Theoretical plate level	Metal accumulation	Metal concentration effluent (μmol/l)
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 μmol/ℓ
Total uptake 99.85%
Effluent metal concentration 0.15 μmol/ℓ

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.

5.4.2 MICROFILTRATION SYSTEM

In the ultrafiltration system the increase in copper in the first samples of series 2 and 3 (Figure 5.14) 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 5.14).

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 initially the influent copper concentration is low and only equates to the concentration of the stock solution after approximately 1 ℓ of influent has been processed; this can be seen in Figure 5.20 by the initial low copper concentration in the effluent.

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 determine 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 disaggregation 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.

CHAPTER 6

APPLICATIONS OF BIOSORPTION OF HEAVY METAL CATIONS BY YEAST BIOMASS

6.1 INTRODUCTION

The mechanisms of heavy metal accumulation by yeasts which have been investigated in this study have an important industrial implication since the removal of heavy metals from industrial effluents is the ultimate aim of heavy metal biosorption research. Although the recovery of heavy metal ions from industrial and mining wastewaters has not been thoroughly investigated, the removal and recovery of these metal ions by mocrobial (yeast) biomass has certain advantages above inorganic methods. These advantages include high metal binding capacity, high selectivity of the microorganisms for certain compounds and most importnatly the low maintenance costs (Rohricht, *et al*, 1990).

However, a number of questions still need to be answered with regard to the practical applications of biomass for this purpose. These include : what ambient conditions could be expected in the wastewater, what properties must the biosorbent possess for it to be effective, etc?

The aim of this section of the present study was to examine examples of possible industrial usage of yeast biomass and to conduct a preliminary investigation on the possible limitations of its use.

6.2 MATERIALS AND METHODS

6.2.1 PREPARATION OF YEAST CELLS

This was done as described in Chapter 2.

6.2.2 PREPARATION OF THE GRANULAR BIOSORBENT

This was prepared by mixing 100 ml wet mass of yeast with an equal volume of 2 mol/ℓ 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. At low magnification the granules appear to have a rough surface. At higher magnification it becomes obvious that the material has an extensive surface area available for metal adsorption. At yet higher magnification flakes of material can be observed which could provide a large surface area for cation adsorption.

6.2.3 ZINC REFINERY EFFLUENT STUDIES

EFFLUENT ANALYSIS

Metal Ions:

Metal concentrations in the effluent were determined by atomic absorption spectroscopy.

Sulphate (SO_4^{2-}) Analysis:

The Zinc Refinery wastewater were analysed for SO_4^{2-} according to the method as described in the Leather Industries Research Institute (LIRI) Technologies, Introductory Course in Wastewater Management manual (1990). The method consisted of adding a solution of barium chloride to a hot solution of the sulphate which is slightly acidified with hydrochloric acid. 5 to 20 ml of a filtered sample was pipetted into a 250 ml Erlenmeyer flask and 50 ml of distilled water was added. The flask was placed on a steam bath and 3 drops of concentrated HCl were added. When hot, sufficient 10% $BaCl_2$ (10-20 ml) was added to precipitate out all the sulphate. A few more drops were added after the precipitate has settled to ensure complete precipitation of the sulphate. This solution was kept on the steam bath for 60 minutes and while hot, filtered through a Whatman No. 42 filter paper. This was washed 3 times with hot distilled water and allowed to drain. The filter paper with the precipitate was placed in a dried and weighed evaporating dish, and dried in an oven at

105°C and then ashed for 4 hours in a 600°C oven. This was then cooled in a desiccator and weighed. The amount of sulphate was calculated by substitution into the following equation:

$$mg/\ell\ SO_4^{-2} = \frac{(\text{wt. dish} + \text{ash} - \text{initial wt. of dish}) \times 0.412 \times 10^6}{\text{aliquot pipetted}}$$

Chloride (Cl^-) analysis:

The Zinc Refinery wastewater was analysed for chloride content using the method as described in the LIRI Technologies Introductory Course in Wastewater Management manual. A suitable aliquot (2-10 ml) of filtered sample was pipetted into a 250 ml Erlenmeyer flask, and 50 ml of distilled water added. The pH of the solution should be 6.5 and above, adjusted with sodium carbonate where necessary. 0.1 N $AgNO_3$ was titrated using potassium chromate indicator (5 drops), and the colour change from yellow to orange-red noted.

Calculation:

$$mg/\ell\ Cl^- = \frac{\text{titration volume} \times 0.003546 \times 10^6}{\text{aliquot}}$$

Fluoride (F^-) Analysis:

The following method was used in assaying the Zinc Refinery wastewater fluoride content, and in the fluoride binding experiments as described by Fries and Getrost (1977). An aliquot of the filtered sample (5 ml) was pipetted into a 10 ml volumetric flask. The following reagents were then added in the given order; 0.2 ml acetate buffer solution, 1 ml reagent solution (Alizarin-3-methylamine-*N,N*-diacetic acid based solution), 1 ml lanthanum nitrate solution and 2 ml acetone, and made up to the mark with milli-Q water. The samples were left to stand in the dark for 150 minutes and then the absorbance measured at 620 nm. The concentration was determined with reference to a standard curve.

Metal Binding Experiments:

The yeast was resuspended in 5 mM PIPES buffer, a biological buffer with negligible metal binding capacity, to concentrations of 0.4 g dry wt/ℓ and 1.0 g dry wt/ℓ using a previously determined absorbance-dry weight standard curve as described by Brady (1992). These

preparations were then used immediately for binding experiments using batch type reactors. Samples were taken at various times to determine the amount of metal bound per mg yeast biomass.

Rate of Metal Uptake:

Yeast suspensions (40 ml) of the desired concentration (0.4 or 1.0 g/ℓ) were placed in 250 ml Erlenmeyer flasks. 1 ml of a metal salt solution (50 x required final concentration) or effluent was added to the flasks, which were then shaken and a 2 ml sample taken immediately. The flasks were placed in an orbital shaker at 25°C. 2 ml samples were taken every 15 minutes for the first 60 minutes and then every 30 minutes for the following 180 minutes and finally after 24 hours, and were analysed by atomic absorption spectrophotometry using previously described methods.

Tangential Flow Microfiltration:

Using the yeast cells, a cross-flow microfiltration experiment was carried out as described by Brady (1992). A polypropylene microfiltration hollow fibre membrane was used, pore size was 0.1 μm. 6.34 g of *S. cerevisiae* was suspended in 1.01 of zinc refinery wastewater, and pumped into the filter cartridge to pack the yeast onto the membrane. 5.01 of the same wastewater was then added to the reservoir in such a way as to maintain a constant volume in the reservoir during the period of the experiment. 10 ml samples were taken every five minutes.

6.2.4 ELECTROPLATING EFFLUENT STUDIES

Equilibration

Following the methods of Tsezos (1988), Kuyucak and Volesky (1988), and others, the yeast biomass was brought into direct contact with the effluent. Initially 0.1 g of unbuffered yeast (wet weight) was suspended per ml of effluent. Equilibration of the yeast-effluent solutions lasted 25 hrs at 25°C with gentle shaking (Tsezos, 1988). At 5 hr intervals 20 ml fractions were removed, centrifuged (3 000 g x 10 min) and the supernatant analysed by atomic absorption spectroscopy to determine metal removal.

In further experiments the concentration of the yeast used in heavy metal bioaccumulation

increased to 0.5 g (wet weight)/ml effluent. During all experiments the pH of the effluent was monitored prior to; on addition of the yeast at the start of incubation, and at the end of the incubation period.

Batch Systems

From the results obtained from the equilibration reactions, a 15 hr period appeared to have the highest efficiency regarding metal removal. The percentage metal removal from a 15 hr continuous batch system was compared to that of three 5 hr batch systems run in series. The latter involved suspending the yeast in effluent (0.5 g/ml) for 5 hours, at 25°C with gentle shaking. After 5 hrs the suspension was spun down (4 000 g x 10 min) and the supernatant added to fresh yeast (also at a concentration of 0.5 g/ml). This procedure was again repeated after 5 hrs. On completion of incubation (i.e. after 15 hrs) both the continuous and series batch systems were spun down and the percentage metal removal determined. Aliquots (10 ml) were removed at 5 hr periods during the series reactions and analysed to monitor the rate of metal accumulation.

Glucose Supplementation

The effect of glucose on metal ion accumulation was investigated. Thirty minutes prior to the addition of effluent anhydrous glucose was added to yeast-water suspensions. A final glucose concentration of 50 mM in solution was used (Perkins and Gadd, 1993). After exposure to glucose, the yeast was recovered by centrifugation and the pellet obtained washed twice with Milli-Q water before being suspended in industrial effluent.

Additional experiments were conducted during which glucose was added directly to the yeast effluent solutions. Trials were conducted during which glucose was added at 0, 5 and 10 hourly intervals to yeast in a continuous batch experiment.

Effect of pH on Metal Accumulation

The optimal pH range for metal ion accumulation by *S. cerevisiae* falls within pH 5-8 range (Brady, 1992). The pH of the three effluents varied, *viz.* pH 2.2, 3.6 and 6.0. Using NaOH

the initial pH's were altered from pH 3.6 to pH 5 for effluent B and that of E from pH 2.2 to pH 6 to determine the effect on metal ion removal. As for previous experiments the pH was monitored for the duration of the experiment.

6.2.5 BIOSORPTION STUDIES WITH NON-VIABLE YEAST BIOMASS

Continuous flow biosorption by columns of granular biosorbent:

Chromatography columns were loaded with aqueous slurries of granular biosorbent (5 g), and metal containing solutions were pumped upwards through the column at a flow rate of 24 ml/hr. The ambient temperature during these biosorption experiments was 20°C. The column volume was 35-45 ml. 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 expressed as parts per million (ppm), i.e. mg of metal per ℓ, a unit of concentration that is commonly used when reporting conditions in wastewater treatment processes.

6.3 RESULTS

6.3.1 ZINC REFINERY EFFLUENT STUDIES

Effluent Analysis

Table 6.1 shows the results of the cation analysis of the zinc refinery wastewater. Mercury, nickel, lead, zinc and selinium levels were relatively high.

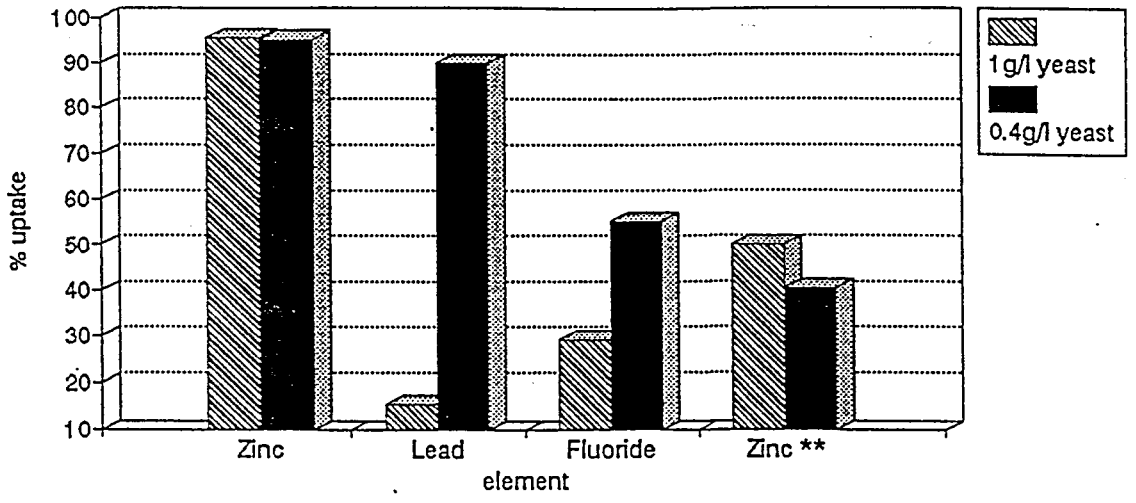
The sulphate concentration of the effluent was 900 mg/ℓ while the concentration of chloride was 323 mg/ℓ and fluoride 0.865 mg/ℓ.

Table 6.1: Metal concentrations in zinc refinery wastewater

Metal	Refinery wastewater (mg/ℓ)
Aluminium (Al)	1.48
Cadmium (Cd)	0.717
Cobalt (Co)	0.410
Chromium (Cr)	2.24
Copper (Cu)	0.103
Iron (Fe)	1.80
Potassium (K)	12.8
Magnesium (Mg)	335
Manganese (Mn)	124
Sodium (Na)	167
Nickel (Ni)	4.11
Lead (Pb)	4.90
Zinc (Zn)	4.03
Mercury (Hg)	21.2
Selinium (Se)	3.94
Silver (Ag)	4.03

Metal Uptake

The ability of the yeast in batch reactors to remove zinc (7 mg/ℓ), lead (7 mg/ℓ) and fluoride (2 mg/ℓ) from solution was examined and a summary of the results is shown in Figure 6.1. It is clear that the yeast cells are very effective in taking up zinc, and at lower yeast concentration also in the uptake of lead and fluoride. Uptake of zinc from the refinery wastewater is also shown in Figure 6.1. The biomass removed 40-50% of the zinc in this effluent.



** = uptake from Zinc refinery wastewater.

Figure 6.1: Uptake of metals from solution by *S. cerevisiae*

Rate of Uptake of Zinc from Zinc Refinery Wastewater

The rate of uptake of zinc (Zn) from zinc refinery wastewater is shown in Figure 6.2. Using two concentrations of *S. cerevisiae* of 0.4 g/ℓ and 1.0 g/ℓ, it can be seen that there is a rapid uptake of Zn in the first 15 minutes followed by slower uptake. From this graph there seems to be a great overall uptake by the 1.0 g/ℓ yeast solution, which is the opposite to previously observed trends. In Figure 6.3 which shows the rate of loss of Zn from the wastewater, the overall Zn ions concentration remaining in solution appears to be the same for both concentrations of yeast.

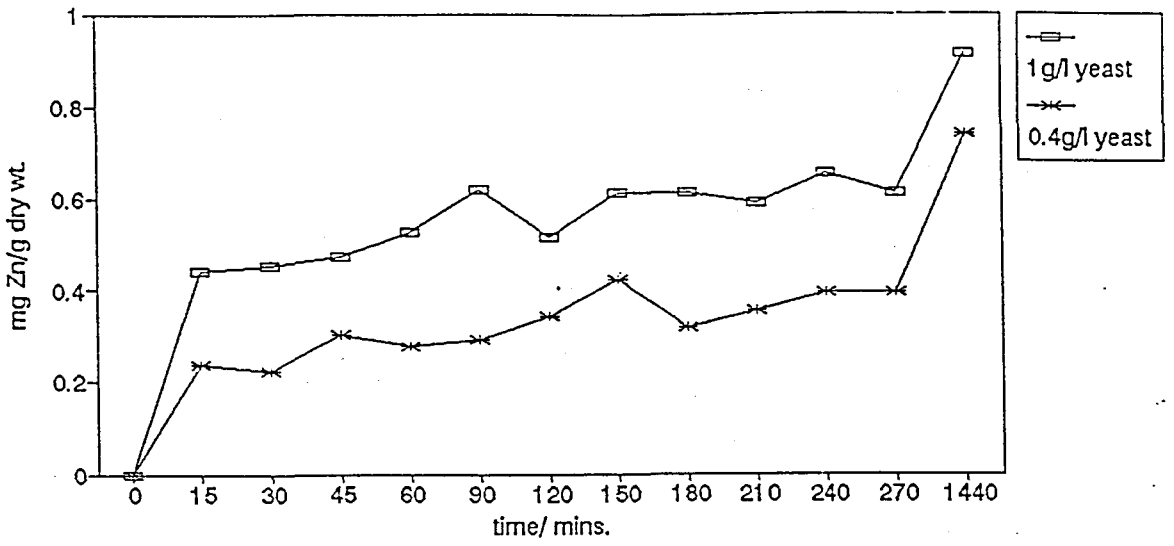


Figure 6.2: Rate of uptake of zinc by *S. cerevisiae* over a 24 hour period after exposure to refinery wastewater.

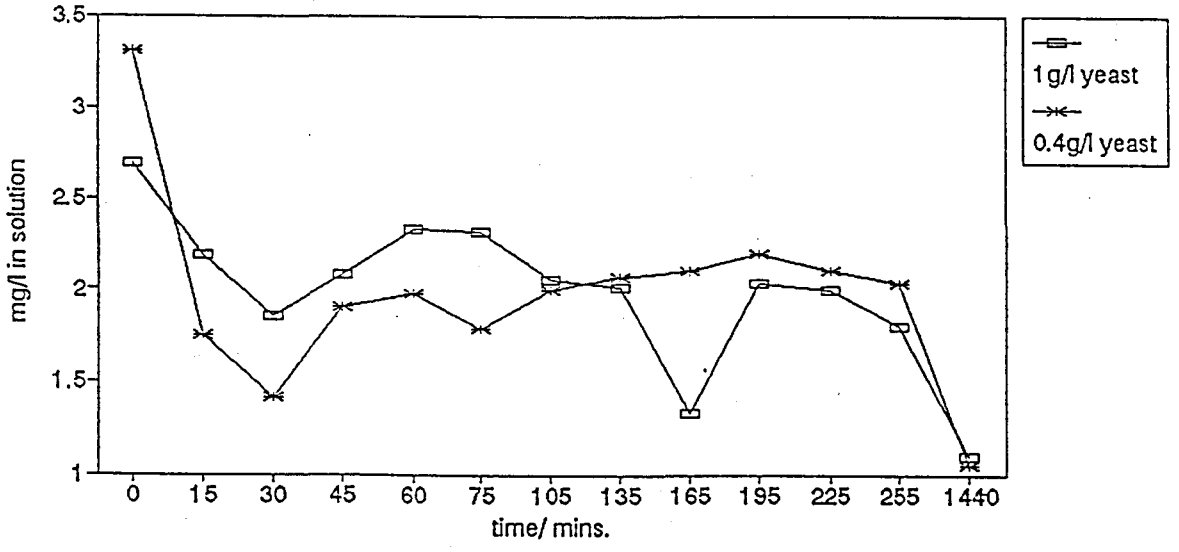


Figure 6.3: Rate of loss of zinc from refinery wastewater with over a 24 hour period

Tangential Flow Microfiltration

In Figure 6.4 it can be seen that membrane-immobilized yeast cells take up zinc and lead from zinc refinery wastewater. The uptake was expressed in terms of a decrease in the amount of the metal ions left in solution. Figure 6.5 shows the rate of loss of fluoride from zinc refinery wastewater, and it shows that there is uptake of fluoride from solution by the immobolised yeast. Figure 6.6 shows the percentages of zinc, lead and fluoride remaining in the zinc refinery effluent after passing it through the immobilized yeast cartridge. 50% of zinc, 10% of lead and 50% fluorude was left in solution.

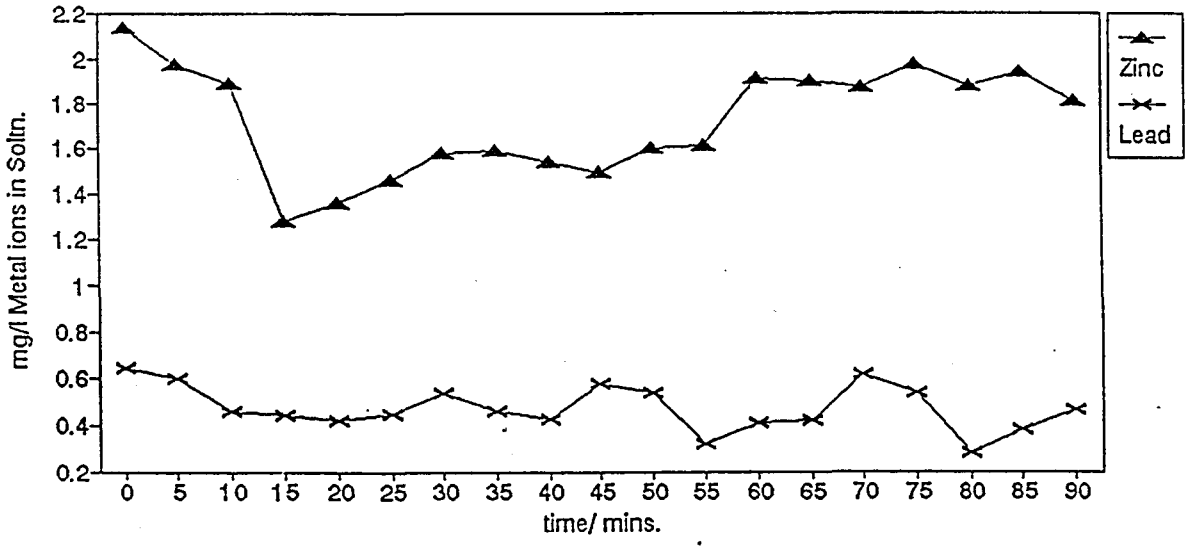


Figure 6.4: Rate of loss of zinc and lead from zinc refinery wastewater using a microfiltration system.

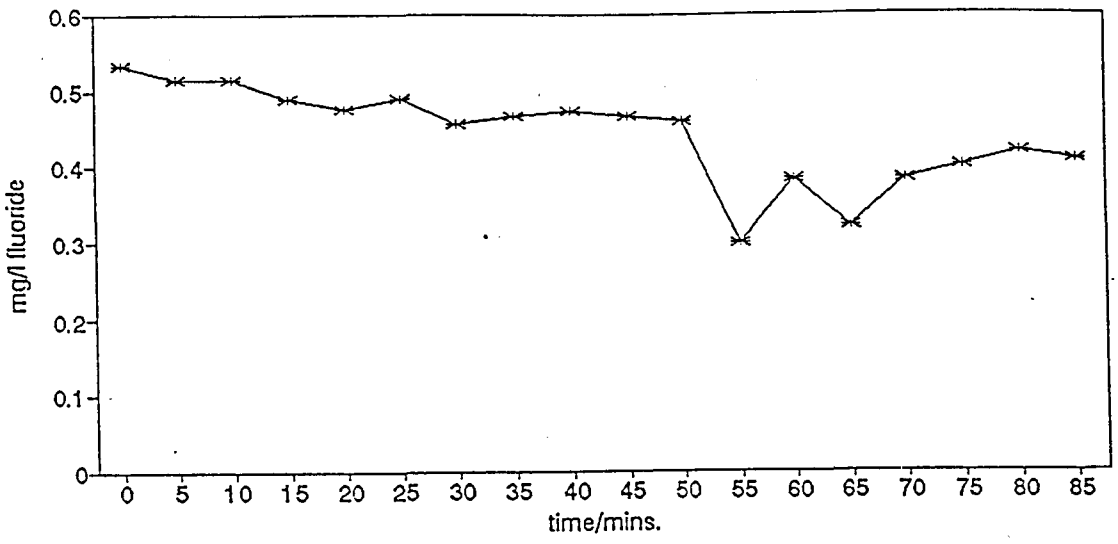


Figure 6.5: Rate of loss of fluoride from zinc refinery wastewater using a microfiltration system.

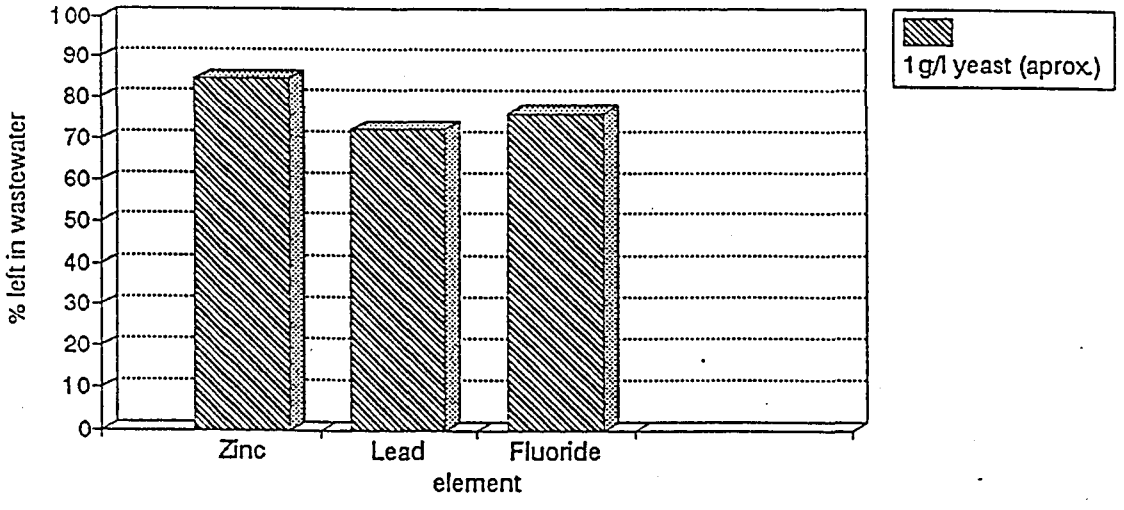


Figure 6.6: Percentage ions remaining in zinc refinery wastewater using a microfiltration system.

6.3.2 ELECTROPLATING EFFLUENT STUDIES

All of the effluents obtained from the electroplating industries contained metal ions in excess of the permitted criteria (Table 6.2).

Table 6.2: Heavy metal profiles of effluents A, B and E in comparison to the median of the permitted metal levels in drinking and dam water (Kempster, *et al*, 1982). The levels are quoted in mg/ℓ

Element	Metal concentration (mg/ℓ)				
	Drinking Water	Dam/River Water	A	B	E
Cd	0.01	0.003	16.0	<0.05	<0.05
Cr	0.05	0.05	<0.05	71	4
Cu	1.00	0.005	0.1	<0.05	30
Ni	0.05	0.05	0.1	<0.05	15.6
Zn	5.00	0.1	260	460	0.3

The removal of heavy metals by yeast biomass in serial batch reactions with shorter contact times (3 x 5 hrs) was superior to that of a single (continuous) batch system (1 x 15 hrs) (Table 6.3).

Table 6.3: Comparison of two batch systems: series (3 x 5 hrs) and continuous (1 x 15 hrs) in the removal of heavy metal cations over a 15 hour period.

Element	Percentage metal removed					
	A		B		E	
	1x15 hrs	3x5 hrs	1x15 hrs	3x5 hrs	1x15 hrs	3x5 hrs
Cd	38.75	87.14				
Cr			31.69	51.41	19.48	53.25
Cu					100.00	96.25
Ni					37.18	63.46
Zn	18.37	66.67	17.39	67.69		

Results obtained from additional experiments indicated metal differences in both the amounts and the rates of accumulation by different metal species. The majority of Cu^{2+} in E was accumulated within the first 5 hrs, after which it levelled off. In comparison, the rate of Ni^{2+} and Cr accumulation was almost linear over time. Similar accumulation patterns were obtained for the removal of Zn^{2+} and Cr from B (data not shown).

The metal mass removed from solution by the yeast biomass (mg metal/g yeast) is dependant on the initial concentration present in the effluent. However, the maximum percentages of metal removed from effluents added to glucose pretreated cells were similar, e.g. 74.65% accumulation of Cr from B (initial conc 71.0 mg/ℓ) and 75.32% of Cr accumulated from E (initial conc 4.0 mg/ℓ) (data not shown).

Selective accumulation of metals by the yeast biomass occurred. Due to differing initial metal concentrations the amount of metal accumulated for comparative purposes was calculated as a percentage of the initial concentration. Based on these calculations the order of preference of accumulation of metals by yeast biomass was :

$$Cu >> Cd > Zn > Ni, Cr$$

Effect of Glucose and pH on Metal Accumulation

No buffer was added for the duration of the experiment. The yeast was brought into direct contact with the effluent. Although the initial pH's varied, *viz.* pH 6.0 (A), pH 3.6 (B) and pH 2.2 (E) the yeast had a buffering capacity, either raising or dropping the pH to approximately pH 4.5 - 5.5 for A and B. Although the biomass did have a buffering effect on E, due to the very low initial pH, this effect was not as marked. After 15 hrs the pH had risen to pH 3.7, yet with time it gradually increased even further (to a pH of 4 after 20 hrs, pH 4.3 after 25 hrs and pH 4.5 after 30 hrs).

Adjustment to the starting pH of B and E during serial and expeirments to pH 5 and pH 6 respectively did not result in increased metal ion accumulation compared to the untreated effluent (Figures 6.7, 6.8). However, metal ion accumulation was severely limited at

extremely low pH. When exposed to a 50 mg/ℓ solution of Ni^{2+}, Zn^{2+}, Cu^{2+}, Cr^{2+} and Cd^{2+} at pH 1 and pH 4 the % of metal removed by the yeast biomass at pH 1 was less than that removed from the pH 4 solution. Only 51% of the Cd removed from the pH 4 solution was removed from the pH 1 solution, 15% of the Cr, 65% of Cu, 61% of Ni and 25% of the Zn. The bioaccumulation mechanisms for Cu and Ni appears to be the least pH sensitive.

Pretreatment of yeast with glucose had a marked effect on metal ion removal from solution. The levels of residual metal ions of treated effluent were lower than those of untreated effluent (Figures 6.7, 6.8, 6.9). However, direct addition of glucose to the biomass-effluent solution had no effect on the removal of metals (Table 6.4).

Table 6.4: Effect of glucose on the removal of heavy metal cations from Effluent A by yeast biomass. The final glucose concentration was 50 mM

Glucose treatment	Metal concentration (mg/ℓ)	
	Zn	Cd
Control	260	16.0
No Glucose	200	9.7
30 min Glucose pretreatment	130	5.8
0 hrs Glucose addition	205	9.8
5 hrs Glucose addition	200	10.0
10 hrs Glucose addition	200	10.0

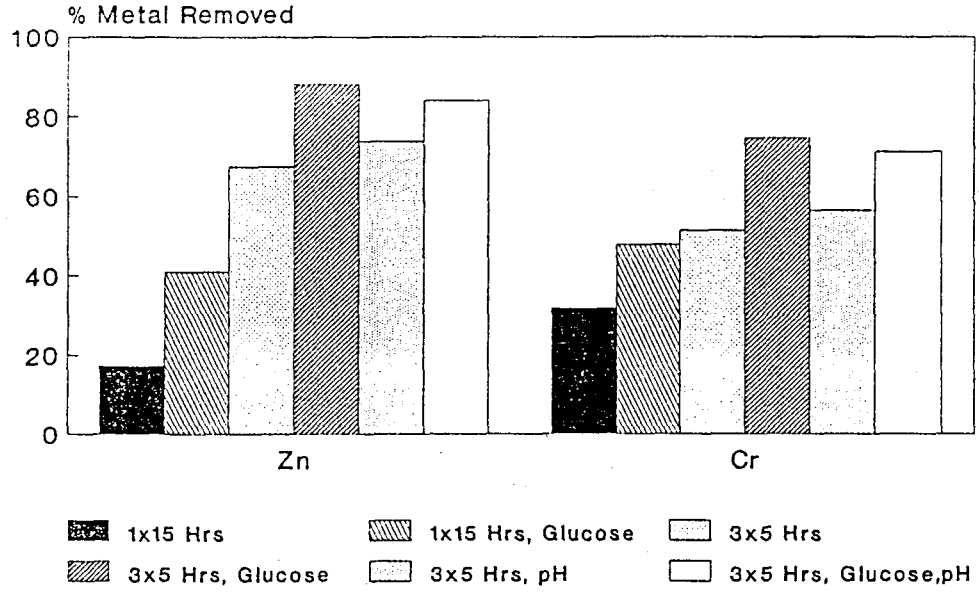


Figure 6.7: Comparative methods for removing metals from Effluent B

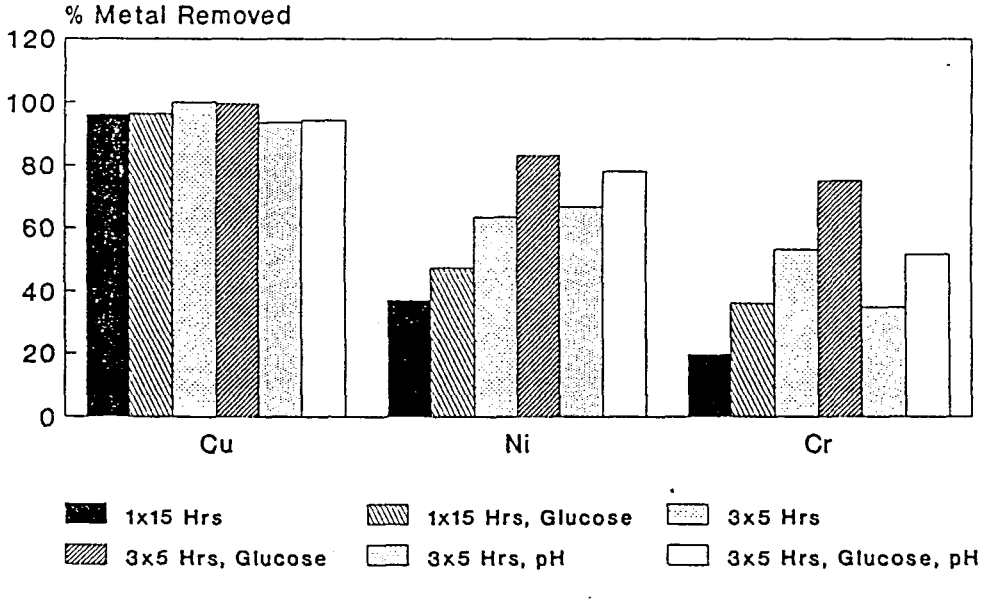


Figure 6.8: % Metal removal from Effluent E by treated and untreated yeast cells in batch systems (1 x 15 hrs, 3 x 5 hrs)

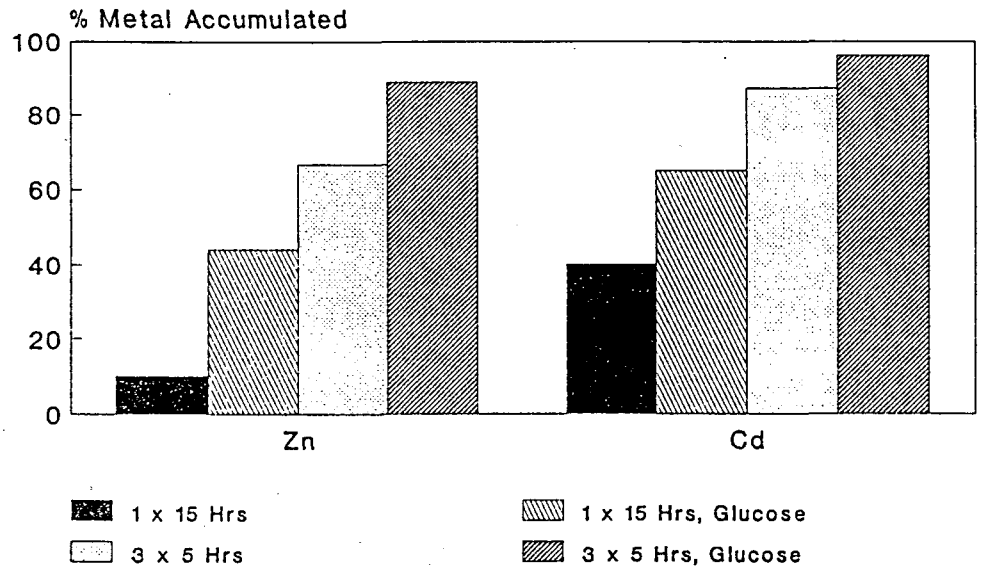


Figure 6.9: Metal removal from Effluent A by treated and untreated yeast cells in batch systems (1 x 15 hrs, 3 x 5 hrs)

6.3.3 BIOSORPTION BY NON-VIABLE YEAST BIOMASS

As a practical assessment of the possible uses of the granular biosorbent prepared by alkali treatment of yeast cells 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 6.10).

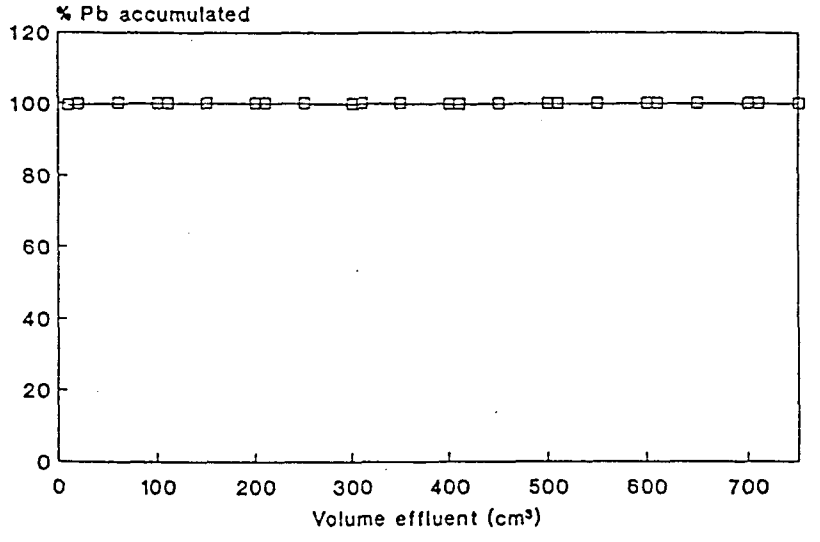


Figure 6.10: Lead accumulation from tapwater by a column of granular biosorbent.

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 is 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 6.11). 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.

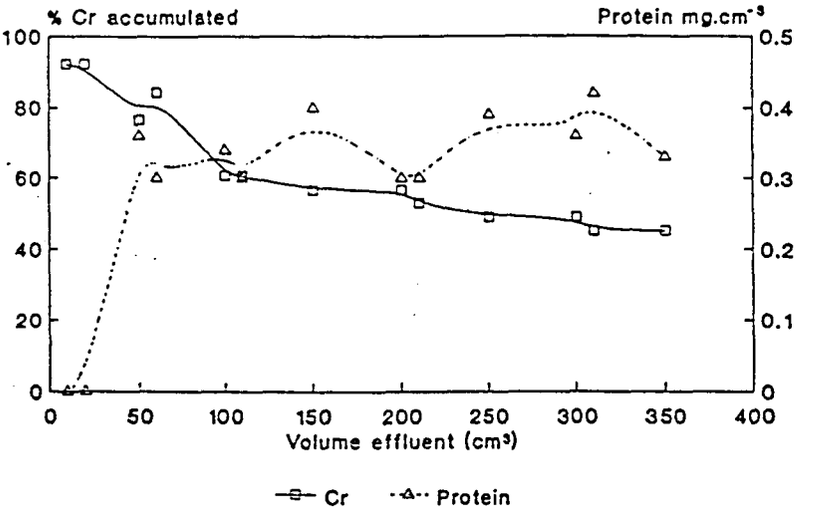


Figure 6.11: Biosorption of chromium from tannery wastewater (post anaerobic digestion) by granular biosorbent. The influent chromium concentration was 2.6 mg/ℓ and the influent pH was 7.51.

In the influent tannery wastewater the concentration of Na was 30 mmol/ℓ, that of Ca^{2+} was 10.3 mmol/ℓ, and that of protein was 0.388 mg/ml. The interference caused by these constituents of the wastewater on chromium accumulation was investigated. Interference of Cr(III) absorption from a 100 mg/ℓ 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 6.12) 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.

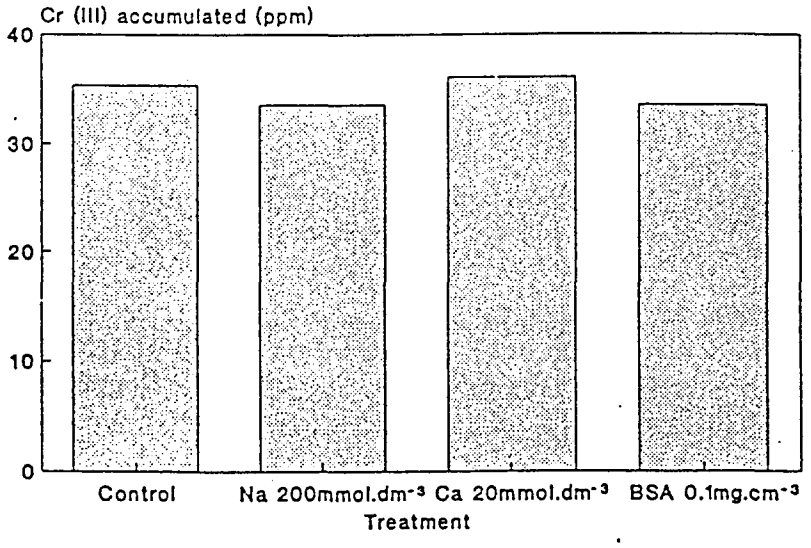


Figure 6.12: Interference of chromium biosorption by granular biosorbent by certain organic and inorganic compounds.

6.4 DISCUSSION

6.4.1 ZINC REFINERY EFFLUENT

It is clear from these preliminary studies that *S. cerevisiae* biomass is able to remove significant amounts of toxic metals such as zinc and lead from a zinc refinery wastewater. The reason that more than 50% of the zinc was not taken up by the cells is probably due to the presence of other metal cations in the refinery wastewater, since it is known that many of these metals compete with zinc for binding to yeasts (Norris and Kelly, 1977).

The toxicity of metal cations in wastewater have been shown to reduce the energy-dependent uptake by the yeast (Norris and Kelly, 1977). 1 g/ℓ yeast solution in the case of this effluent, bound more zinc than the 0.4 g/ℓ solution, which is the contrary to previous observations. This is probably due to the presence of many other cations in solution that compete for available binding sites, and therefore the more yeast the greater the amount of zinc bound.

Although there is no known published literature on fluoride uptake, binding isotherms using *S. cerevisiae* were carried out with a fluoride concentration of 2 mg/ℓ. Fluoride is known to be toxic at high concentrations, and effluents with concentrations above acceptable regulation limits need to be treated. The results of this study again indicate that it is possible to achieve this by use of biomass such as yeast to accumulate the fluoride.

The cross-flow microfiltration (CFM) unit with immobilized yeast did not show high uptake. This may be due to the use of the zinc refinery wastewater to resuspend the yeast cells for packing into the cartridge. During the hour long process of packing the yeast cells onto the membrane, there is time for extensive interaction between the yeast cells and metal ions in solution. When the rest of the wastewater was passed through the cartridge, there were less binding sites available to the metal ions. When packing a column one needs to use milli-Q or a buffer with negligible binding capacity. Further studies are therefore required to refine this system.

6.4.2 ELECTROPLATING EFFLUENTS

The amount of metal removed from the various electroplating effluents was dependant on the initial concentration present in the effluents. Some of these effluents had very high levels of zinc (> 260 mg/ℓ) and the batch reactors with *S. cerevisiae* as the biosorbent were capable of removing approximately 66% of this zinc from the effluent solutions. Chromium at a level of 71 mg/ℓ was removed with an efficiency of 50% while 87% of cadmium from an effluent containing 16 mg/ℓ was achieved. Selective metal accumulation was also achieved.

The data indicate that the bioaccumulation mechanisms for some metals appear to be pH sensitive while others such as those for Cu and Ni are less sensitive to pH. Pretreatment of the cells with glucose increases the removal of efficiency of the yeast cells which suggests that they may require an energy source to maximise metal accumulation. This would presumably be required for internalisation of the metals since earlier studies (Brady, 1992) cell wall binding (biosorption) appear to be energy independant.

6.4.3 NON-VIABLE YEAST BIOSPORTION

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. Other methods of elution will therefore have to be applied or developed for this biosorbent.

CHAPTER 7

CONCLUSIONS

7.1 MECHANISM OF ACCUMULATION OF HEAVY METALS BY YEAST

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 and that outlined in a previous report (Duncan and Brady, 1993), 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.

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 and others (Duncan and Brady, 1993 and Brady and Duncan, 1993a), 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 alter the affinity of the cell wall for metals. However, it is unlikely that such modifications would be economically viable. As the "ion storehouse" of the cell the vacuole also appears to be potentially capable of accumulating vast quantities of metals. A great deal more work is however necessary to determine the exact role of these intracellular organelles in the bioaccumulation of metals.

The release of certain cations which are specifically compartmentalized into the vacuole or cytosol allowed tentative conclusions to be made about the role that this compartment may play in uptake of heavy metals such as copper. There is a slow 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 has facilitated a better understanding of the process of heavy metal uptake and eventual compartmentalization of the accumulated metal.

Thus it can be concluded from these results and those discussed in the previous chapter that yeast cells are capable of accumulating heavy metal cations. The cell wall is the first cellular structure to come into contact with the metals present in the extracellular solution. All cations appear to bind to the wall in a similar fashion and only differ in the amounts bound. Entry into the cell is facilitated by a H^+/antiport system. Subsequent efflux of intracellular physiological cations (Na, K, Mg) occurs in response to the accumulation of some species of heavy metal cations.

Once inside the cytosol, the cations are taken up by the vacuole either along a H^+ gradient, which is created by a V-ATPase H^+/antiport system, or alternatively through permeation of the tonoplast. The heavy metals are sequestered within the vacuole to reduce cellular toxicity.

The abovementioned mechanism has important implications in industry where already it has proven capable of removing the majority of heavy metal ions from effluents containing high concentrations of these metals.

7.2 IMMOBILIZATION

Column 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.

The use of cross-flow microfiltration units in bioaccumulation processes also 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.

The full potential of CFMF technology in bioaccumulation is not presently 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 minimize 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 foreseen.

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.

7.3 EFFLUENT TREATMENT

Wastewaters often contain several different metal ions and the specification of these metal ions is important in metal uptake processes. The high concentrations of some of these metal ions such as that found in the mining and electroplating effluents, may affect the uptake of metal ions of interest by competing for common binding sites. This could consequently account for the lower percentage uptake of zinc and lead from the mining effluent when compared to solutions containing only the metal of interest.

However, it is clear from the results of the studies using both live and non-viable yeast cells, that there is a great deal of potential for the use of biosorbents for heavy metal removal from effluents and wastewaters. Immobilization of biomass either in columns, gel matrix type systems or in microfiltration systems is required for the application of bioaccumulation in real life situations. Some promising progress has been made in this regard but the systems will require refining and further experimentation is needed before systems for particular applications can be fully defined. Such experimentation will facilitate the development of pilot scale treatment plants in the near future.

As far as the non-viable cell biosorbents are concerned, either in granular form or as soluble flocculators and chelating adjuncts, they 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.

The need for a cheap, non-polluting, biological method for the treatment of wastewater for reuse is a worldwide concern and needs to continue receiving particular attention.

7.4 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 mg/ℓ and discharge concentrations are required to be less than one mg/ℓ (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 bioaccumulation 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.

In 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.

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 system 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 together with earlier work in our laboratory 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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