THE REMOVAL AND RECOVERY OF TOXIC AND VALUABLE METALS FROM AQUEOUS SOLUTIONS BY THE YEAST *Saccharomyces cerevisiae*

A thesis submitted in the fulfilment of the
requirements for the degree of

DOCTOR OF PHILOSOPHY
in the
Department of Biochemistry and Microbiology
Faculty of Science
Rhodes University

by

Brendan Shane Wilhelmi

November 1997

This thesis is dedicated to

Loana Wilhelmi and

Keith and Margaret Winter

PUBLICATION LIST

Parts of this thesis published as:

Wilhelmi B.S. and Duncan J.R. (1995) Metal recovery from *Saccharomyces cerevisiae* biosorption columns, *Biotechnol. Lett.* **17**, 1007-1012.

Wilhelmi B.S. and Duncan J.R. (1996) Reusability of immobilised *Saccharomyces cerevisiae* with successive adsorption-desorption cycles, *Biotechnol. Lett.* **18**, 531-536.

Duncan J.R., Brady D. and Wilhelmi B.S. (1997) Immobilization of yeast and algal cells for bioremediation of heavy metals. In: *Methods in Biotechnology, vol 2: Bioremediation protocols*, Sheehan D. (ed), Humana Press Inc., Totowa, NJ, 91-97.

Duncan J.R., Stoll A., Wilhelmi B.S., Zhoa M. and van Hille R. (1997) The use of algal and yeast biomass to accumulate toxic and valuable heavy metals from wastewater, *Water Research Commission Report*, # 616.

Conference presentations:

Wilhelmi B.S. and Duncan J.R., The removal and recovery of metals from aqueous solutions by immobilized *Saccharomyces cerevisiae*, *Proc. 13th SA Biochemical Society Congress*, Bloemfontein, 1995.

Duncan J.R., Brady D., Stoll A. and Wilhelmi B.S., Heavy metal removal from industrial effluents by immobilised biomass, *Proc. 13th Intl. Congress on Biohydrometallurgy*,. Chile, 1995.

Wilhelmi B.S. and Duncan J.R., Bioremediation of copper from water by immobilised *Saccharomyces cerevisiae*. *Proc. Biotechnology for Africa 95*, Pretoria 1995.

Duncan J.R., Wilhelmi B.S., Stoll A., Makhale T.C. and Brady D., Heavy metal removal from industrial effluents by yeast and algal biomass, *WISA Proc.*, Port Elizabeth, 1996.

Wilhelmi B.S. and Duncan J.R. Bioremediation of mixed metal aqueous solutions using brewer's yeast *Saccharomyces cerevisiae*, *Proc. Biotech. SA '97*, Grahamstown, 1997.

Wilhelmi B.S., Cross R., Pinchuck S., Neethling J.H., Douglas A and Duncan J.R. Bioremediation of copper and iron by *Saccharomyces cerevisiae*: TEM energy dispersive x-ray analytical investigation of metal deposition sites, *Proc. Microscopy society of Southern Africa*, 1997.

Abstract

This project considered the use of the yeast *Saccharomyces cerevisiae* as a biosorbent for the removal and recovery of a range of metals from contaminated waters. *S. cerevisiae*, as a biosorbent, has the potential to provide a cost effective, selective and highly efficient purification system.

Initial studies focused on metal accumulation by an immobilized baker's *S. cerevisiae* biosorbent. The parameters affecting metal uptake were investigated, these included metal concentration, time and solution pH. Metal uptake was rapid. Gold and cobalt reached saturation within 5 min of contact with the biosorbent in batch reactors. Copper, zinc, nickel, cadmium and chromium reached saturation within 30 min of contact. Metal accumulation was pH dependent and was generally unaffected at a solution pH $\geq$ 4, and was substantially decreased at pH $\leq$ 2. The exception was gold which was preferentially accumulated at a solution pH of 2. The immobilized baker's yeast accumulated metals with maximum binding capacities in the order of gold > cadmium > cobalt > zinc > copper > chromium > nickel.

A rapid method to assess metal recovery was developed. Bioaccumulated metal was efficiently recovered using dilute mineral acids. Copper recovery of $\geq$ 80 % was achieved by decreasing the solution pH of the reaction mixture to 2 with the addition of nominal quantities of HCl, H_2SO_4 or HNO_3. Adsorption-desorption over 8 cycles had no apparent adverse effect on metal uptake or recovery in batch reactors. Transmission electron microscopy showed no evidence of damage to cells used in copper adsorption-desorption investigations.

Biosorption columns were investigated as bioreactors due to their application potential. The metals investigated were effectively removed from solution. At a saturation threshold, metal uptake declined rapidly. Most metals investigated were desorbed from the columns by eluting with 0.1 M HCl. Initially recoveries of copper, cobalt and cadmium were as high as 100 %. Desorbed copper, zinc, cadmium, nickel and cobalt were concentrated in 10 to 15 mℓ of eluent, representing up to a 40 fold decrease in solution volume. Cadmium, nickel and zinc uptake increased with the second application to the columns. Initial accumulation of gold and chromium was 42.2 μmol/g and 28.6 μmol/g, however, due to the low recoveries of these two metals, a second application was not investigated. Copper was applied to a single column for 8 consecutive adsorption-desorption cycles. Uptake increased from an initial 31.3 μmol/g to 47.8 μmol/g at cycle 7. The potential for selective metal recovery was demonstrated using two biosorption columns in series. Copper was accumulated and recovered most efficiently. Zinc, cobalt and cadmium were displaced to the second column. Copper bound preferentially to zinc at a ratio of 6:1. Copper bound preferentially to cobalt at a ratio of 4:1. Cadmium was only displaced at a ratio of 2:1.

The successful transfer of the bioremediation technology from the laboratory to an industrial application has yet to be realized. Bioremediation of a Plaatjiesvlei Black Mountain mine effluent, which contained copper, zinc, lead and iron, was investigated in this project. The removal of the metals was most effective at pH 4. A combined strategy of pH adjustment and bioremediation using immobilized *S. cerevisiae* decreased the copper concentration by 92.5 %, lead was decreased by 90 % and zinc was decreased by 60 %. Iron was mostly precipitated from solution

at pH ≥ 4. An ageing pond at the mine with conditions such as; pH, water volume and metal concentration, which were more conducive to biological treatment was subsequently identified. The investigation indicated a possible application of the biomass as a supplement to chemical remediation.

The metal removal capability of a waste brewer's yeast was subsequently investigated. A yeast conditioning step increased metal uptake up to 100 % and enhanced reproducibility. Metal removal from solution was rapid and pH dependent. The metals were efficiently removed from solution at pH ≥ 4. Uptake was substantially inhibited at pH≤ 3. The waste brewer's yeast accumulated metals with maximum binding capacities in the order of copper (25.4 μmol/g) > lead (19.4 μmol/g) > iron (15.6 μmol/g) > zinc (12.5 μmol/g). No correlation between cell physiology and metal uptake was observed. Uptake of the four metals was confirmed by energy dispersive X-ray microanalysis.

The interference of lead, zinc and iron on copper uptake by the waste brewer's yeast, and the interference of copper on the uptake of lead, zinc and iron was investigated. Maximum copper uptake was not decreased in the presence of lead. The B_{max} remained constant at approximately 25 μmol/g. The dissociation constants increased with increasing lead concentrations. Lead bioaccumulation was significantly decreased in the presence of copper. The type of inhibition was dependent on the initial copper concentrations.

Zinc had a slight synergistic effect on copper uptake. The copper B_{max} increased from 30.8 μmol/g in a single-ion system to 34.5 μmol/g in the presence of 200 μmol/ℓ of zinc. Zinc uptake was severely inhibited in the presence of copper. The maximum uptake and dissociation constant values were decreased in the presence of copper, which suggested an uncompetitive inhibition. The affinity of copper was substantially higher than zinc. The presence of higher levels of copper than zinc in the yeast cells was confirmed by energy dispersive microanalysis.

Copper uptake was decreased in the presence of iron, with the copper B_{max} being decreased from 25.4 μmol/g in a single-ion system to 20.1 μmol/g in the presence of 200 μmol/ℓ iron. Iron B_{max} values remained constant at 16.0 μmol/g. Combined biosorption and EDXA results suggested the iron bound at a higher affinity than copper to the cell wall. Total copper removal was higher as larger quantities of copper were deposited in the cell cytoplasm.

Metal removal from the Plaatjiesvlei effluent by free cell suspensions of the waste brewer's yeast was satisfactory. Copper levels were decreased by 96 %, iron by 42 %, lead 25 % and zinc 2 %. Waste brewer's yeast is a cheap source of biomass in South Africa, and could potentially provide the basis for the development of an innovative purification system for metal-contaminated waters.

CONTENTS

Page

List of Figures

List of Tables

ACKNOWLEDGEMENTS

To the staff members of the Biochemistry and Microbiology Department, Rhodes University, my sincere thanks for generous assistance throughout my post-graduate studies. In particular, I would like to thank Professor J. Duncan for his supervision of this project.

To my colleagues, Anita Stoll and Rob van Hille, thank-you for the assistance and friendship. To my fellow Rhodes squash players, thank-you for the magnificent team spirit and for enhancing my Rhodes University experience.

Most important, a special thank-you to Samantha for her understanding, encouragement and loving support through the last few years, and to my family for their continuous encouragement and support.

I am indebted to Robin Cross, Shirley Pinchuck, Professor J. Neethling and Alistair Douglas for assistance with the electron microscopy and energy dispersive X-ray analysis.

To Rodney Smith and Professor J. Neethling for proof-reading this thesis.

Financial support for the project from the Water Research Commission, the Foundation for Research and Development and Gold Fields is gratefully acknowledged.

Abbreviations

EDAX	Energy dispersive X-ray system trade name
EDXA	Energy dispersive X-ray analysis
PIPES	1,4 piperazinediethanesulphonic acid
TEM	Transmission electron microscopy

CHAPTER I

LITERATURE REVIEW

1.1 Introduction

Increasing levels of heavy metals are being released into local water supplies from industries such as mining, battery manufacture, leather tanning and electroplating. The effects of metals in ecosystems vary considerably, with toxic metals in water resources presenting unacceptable health risks (White *et al*, 1995). The negative effect of these metals on the environment has promoted research into the development of cost effective waste water purification systems (Volesky, 1987; Gadd, 1989). Microorganisms have the ability to bind and concentrate metals from aqueous solutions, with both viable and non-viable cells having the ability to function as efficient biosorbents (Brady and Duncan, 1994a; Brady *et al*, 1994b). Bioremediation could offer an environmentally advantageous technology over conventional chemically orientated metal removal methods. A second motivation for the study of metal-microbial interactions is the increased consumption of metals, which has led to a growing scarcity and an increased value of metals (Lundgren *et al*, 1986). The process has the potential of providing a selective removal and recovery process for both toxic and valuable metals from contaminated waters. Metal concentration in waste streams are often too low for treatment by traditional chemical methods but too high for discharge into the environment. Metal removal by biosorbents could provide a particularly apt strategy for decontamination of these high volume low metal concentration effluents (Guibal *et al*, 1992).

Research emphasis has largely been placed on the identification of biosorbents likely to be the most cost effective. A wide variety of novel biomaterials have been suggested and investigated for the potential use in metal bioremediation such as sawdust preparations from pine and cedar for nickel removal (Chatterjee *et al*, 1996); rice hulls, an unusable by-product from commercial rice harvesting were suggested Roy *et al* (1993) and sphagnum moss peat for the removal of hexavalent chromium (Sharma and Forster, 1993). Most of

the biosorbents under investigation are however the bacteria, fungi or algae produced by industrial processes or which are easy and cheap to propagate in sufficiently large quantities. The potential benefits of metal removal by biological systems include the use of naturally abundant biosorbents, the ability to treat large volumes of water, the selective removal and recovery of metals, the relatively low infrastructure and operational costs and a substantial decrease in volume of toxic waste (Wilde and Benemann, 1993).

1.2 Potable water supply in South Africa

South Africa experiences considerable inter-annual variability with drought and wet years occurring regularly (Mason and Joubert, 1995). Growth of the population, urbanisation and industrialisation serve to increase the demand for water. By 2025, 90 % of population growth will take place in urban areas, increasing the demand of suitable quality water for domestic, municipal and industrial use. In the developed world, industry accounts for 40 % of total water used, while in developing countries such as South Africa this consumption is less than 10 %. Thus industrial development will lead to further increases in demand and a decline in water quality and necessitate clear and enforceable regulations for controlling pollution (Giacasso, 1996).

Water supply is related to a basic increase in quality of human life, and an aim of the reconstruction and development program in South Africa is to supply everyone in the country with 25 ℓ of potable water per day within a radius of 200 m of their residence. Approximately 30 % of South Africans do not have access to adequate water supply. Local authorities are the primary service providers and are confronted by a massive challenge extending these services to previously disadvantaged inhabitants with the historical background of neglect, sabotage and boycotts (Lotter and Pitman, 1996). The cost of supplying the 12 million people in South Africa who at present do not have clean drinking water is estimated to be between R 1 to R 10 billion* (Stephenson, 1995). The careful management, conservation of resources and ensuring of effective measures against pollution have been identified as areas of utmost consideration by the relevant authorities.

* $1 = R4.80

Uniform effluent standards have served the purpose of limiting the rate of water quality deterioration, focussed attention on pollution and resulted in improvements to wastewater treatment technology and water management. Changes in urbanisation patterns with the establishment of large low-cost high density urban areas of previously disadvantaged population groups and intense industrial activity, particularly in the Gauteng region, have impacted negatively and caused a gradual deterioration in the quality of South Africa's potable water. The policy of the Department of Water Affairs to pollutants posing the greatest threat is a precautionary approach, aimed at decreasing or preventing these inputs into the water environment (van der Merwe and Grobler, 1990).

The Gauteng province represents South Africa's most populated region. Water for this region of 16 815 km^2, which comprises 25 % of South Africa's total population, is supplied by the Rand Water Board. Water quality is grouped into three domains which are taken into consideration by the water supplier. The first concerns the physical and chemical components in the water. The second is the aesthetic requirements which include the appearance, taste and odour of water. Current technology and law do not enforce these standards, but they are important for consumer satisfaction. The third water quality concern is the long term effects of small doses of potentially harmful substances (Meintjies and Whitehead, 1996). Rand Water obtains its water from two main sources, the Vaal Dam which has a relatively low salt concentration and a high turbidity, and the Vaal river barrage which is a highly mineralised source containing recycled sewage effluent and high sulphate waters from the gold mining industry (Osborn, 1989). The potable water is produced through the removal of suspended matter and other solids in raw water by the addition of chemical coagulants and flocculants, chemical stabilisation, filtration and disinfection. The suspended matter settles out in the form of a sludge at a rate of up to 1000 tons per day in sedimentation bays. The sludge is conditioned, thickened further and disposed (Baxter, 1996).

Metal surveys of rivers near densely populated urban areas showed elevated levels (up to one hundred times higher than background samples) of copper, lead, zinc, cobalt, nickel

chromium and mercury. The increased metal concentrations were directly attributed to urban run-off (Talbot *et al*, 1985; Watling *et al*, 1985). The bioremoval of these metals released into the environment by human activities is the focus of this project due to the toxicity and potential value of the metals.

1.3 Metal contamination of the environment

Potable water, by definition, should be fit for human consumption. Drinking water quality criteria represents the maximum level of a contaminant at which the water can be consumed with relative safety. Although standards are influenced by practical and political considerations, they should assure against the occurance of adverse effects to public health and the general environment with a large margin of error. Maximum permissible limits (maximum limit for insignificant risk) as proposed by Kempster and Smith (1985); and Aucamp and Vivier (1987) are presented in Table 1.1 (Pieterse, 1989). The term 'metals' is used generally to describe the highly toxic transition metals such as cadmium, lead and mercury, those toxic at high concentrations such as copper and cobalt, the precious metals and radionuclides (Volesky and Holan, 1995; Summers 1992; White and Gadd, 1990). Metals of particular interest to this study are those of high toxicity and common occurrence in industrial waste waters.

Almost all industries discharge at least one trace metal into the soil or water. An estimated 5 % of the total annual production of metals used in primary manufacturing (Mn, Mo, Ni and V) are discarded in the biosphere. The wastage of Cd, Cu, Pb, Cr and Zn which have significant industrial applications, is as high as 10 % of annual production. The major sources of metal pollution in aquatic ecosystems are domestic wastewater effluents (As, Cu, Mn and Ni), coal-burning power plants (As, Hg and Se), non-ferrous metal smelters (Cd, Ni, Pb and Se), dumping of sewage sludge (As, Mn and Pb) and discharge of industrial effluents into lakes and rivers. The major route of lead entry into natural waters is through the atmosphere. The redistribution of increasing amounts of toxic metals into the biosphere and their inevitable transfer to the human food chain is an important environmental issue which may lead to unknown future health risks (Nriagu and

Table 1.1: Proposed drinking water criteria for South Africa (maximum permissible limits).

Determinant	Units	Maximum limit
Alkalinity (as $CaCO_3$)	mg/ℓ	650
Aluminium	μg/ℓ	500
Boron	mg/ℓ	2
Cadmium	μg/ℓ	20
Calcium	mg/ℓ	200
Chlorine	mg/ℓ	600
Chromium	μg/ℓ	200
Cobalt	μg/ℓ	500
Copper	mg/ℓ	1
Cyanide	μg/ℓ	300
Gold	μg/ℓ	5
Iron	mg/ℓ	1
Lead	μg/ℓ	100
Manganese	mg/ℓ	1
Mercury	μg/ℓ	10
Nickel	μg/ℓ	500
Nitrate	mg/ℓ	10
pH	pH units	5.0 - 9.5
Phosphate	mg/l	0.2
Potassium	mg/ℓ	400
Radium	μg/l	1
Silver	μg/ℓ	50
Sulphate	mg/ℓ	600
Temperature	°C	≤30
Thorium	μg/l	0.5
Uranium	mg/ℓ	4
Zinc	mg/ℓ	5

(Kempster and Smith, 1985; Aucamp and Vivier, 1987, cited Pieterse, 1989; Kempster *et al*, 1980).

Pacyna, 1988).

Toxicity of chemical substances to humans is divided into two broad classes: (1) acute or chronic and (2) carcinogenic, although the process of estimating the risk of cancer in humans by exposure to potential chemical carcinogens is a focus of controversy among toxicologists (Pieterse, 1989). Toxicity caused by heavy metals in the environment is more easily determined by toxicity evaluation methods as described Schubauer-Berigan *et al* (1993). Most metals are toxic and sufficiently high concentrations and examples of toxic effects are cited below.

Copper, which is a trace element, is toxic to humans and most microorganisms at high concentrations (Imahara, 1978). Systemic effects in humans such as haemolysis, liver and renal damage have been reported after ingestion of large amounts of copper salts (Piscator, 1979). Chromium (VI) is a powerful carcinogenic agent that modifies the DNA transcription process causing chromosomic aberrations (International Agency for Research in Cancer, 1982; cited from Pĕrez-Candela *et al*, 1995). Nickel is toxic mainly because of its role as an antagonist to essential metals. At high concentrations nickel ions interact with the cellular components, such as; organic acids, nucleotides, amino acids and phospholipids, which interferes with physiological and biochemical processes of cells (Joho *et al*, 1995). The carcinogenicity of nickel has been confirmed in mammals (Reith and Broegger, cited from Bordons and Jofre, 1987). The effects of cadmium in experimental animals include liver and renal dysfunctions, hypertension, chromosomal aberrations and tumour induction. Absorbed cadmium is mainly stored in the liver and kidneys and as with lead and mercury, it is excreted extremely slowly. Cadmium has a half-life of approximately 20 years in human beings (Friberg *et al*, 1979). Long term effects include emphysema (air inhalation) and renal tubular dysfunction (daily intakes of above 300 μg). Cadmium was responsible for the development of Itai-Itai disease after cadmium waste was dumped into Japan's Jinstu river by the Kamioka mining company (Babich and Stotzky, 1980). Cobalt is classified as possibly carcinogenic to humans by the International Agency for research in cancer, although epidemiological data is not

conclusive. In *S. cerevisiae*, cobalt chloride is mutagenic to mitochondrial genes (Beyersman and Hartwig, 1992).

Metal toxicity is dependent on solution speciation as well as different physical, chemical and biological factors (Trevors *et al*, 1986). The physicochemical characteristics of an ecosystem into which metals are discharged determine the chemical speciation forms and thus the bioavailability and toxicity. The effect of metals on the activities of natural microbial populations are the inhibition of primary productivity, nitrogen fixation, mineralization of carbon, nitrogen sulphur and phosphorus, and enzyme synthesis. Characteristic of metal contaminated environments are a decrease in species diversity and the development of metal resistant microbial populations (Babich and Stotzky, 1985).

The trend towards combining industrial and municipal wastes has important implications for the operation of plants using activated sludge systems. Lamb and Tollefson (1973) reported a 90 % decrease in the conversion of organic matter due to the toxic effects of copper (II) at 5 mg/ℓ on the aerobic and anaerobic bacteria in sewage plant effluent. Chromium oxide decreases conversion efficiency by 50 %. Traditional water treatment systems may in future require pretreatment of metal contaminated wastewaters. Bioremediation using microorganisms with high metal tolerance and uptake capacities may provide an effective pretreatment system.

1.4 Metal removal from water

Biosorption of a variety of metals has been demonstrated by a wide range of microorganisms. The ability to bind and concentrate allows for the recovery of these metals and the reincorporation into the industrial process.

1.4.1 The biosorbents

Different species of fungi, algae and bacteria have successfully been investigated for their metal sequestering capabilities. The biosorbents under investigation are often the waste products of industrial fermentation processes which provide large quantities of biomass

at a low cost. Marine algal biomass types available in abundance in the oceans have been investigated and have high metal removal capacities (Holan and Volesky, 1994). Tsezos and Volesky (1981) have demonstrated the efficiency of uranium uptake by *R. arrhizus* to be 2.5 and 3.3 times higher than traditional ion exchange resin and activated carbon, respectively. Bioremoval of thorium by the biomass was 20 times and 2.3 times higher compared to the above conventional systems, respectively.

Microorganisms may also be selected from their natural environments (usually metal contaminated sites) for their specific metal removal capabilities which may allow high and selective recovery of target metals. Pümpel *et al* (1995) describe a rapid agar screening method enabling the selection of metal-accumulating microorganisms from mixed colonies. The basic principle is the growth of colonies on agar plates which are then overlayed with a further agar layer containing the metal of interest. After incubation the metal can be visualized by precipitation or optical effects due to the interaction with the metal. The isolation technique for metal-accumulating microorganisms is effective, rapid and material costs are minimal. The challenge, once a microorganism has been selected, is its successful and cost effective propagation.

1.4.2 Microorganisms and metal removal

Metal uptake by many different biosorbents has been reported extensively, however, comparison of results between different laboratories is difficult because of the many variables involved and the inconsistency of interpretations. The following summary briefly illustrates the diversity and potential of different biosorbents and their ability to remove and concentrate a range of metals from aqueous solutions.

Cadmium uptake by living *Saccharomyces cerevisiae* was 0.62 mmol/g, while only 0.18 mmol/g for inactive cells (Volesky *et al*, 1992). Living cells of *S. cerevisiae* removed up to 0.59 mmol/g uranium, 0.47 mmol/g zinc with the maximum saturation for copper removal not being attained. The uptake capacity of metals by *S. cerevisiae* was strain dependent and dead cells removed up to 40 % more uranium and zinc than corresponding

live cultures (Volesky and May-Phillips, 1995). Lesuisse *et al* (1987) demonstrated the ability of *S. cerevisiae* cells to utilise iron in the ferric (Fe^{3+}) state by reducing it to the aqueous soluble ferrous form which is internalised to meet cellular iron requirements. Uranium reached a concentration of 15 % of cell dry weight of both *S. cerevisiae* and *Pseudomonas aeruginosa* (Strandberg *et al,* 1981). Zinc accumulation by denatured mycelial fungal species *Rhizopus arrhizus*, *Mucor meihei* and *Penicillium chrysogenum* was 0.57, 0.52 and 0.33 mmol/g (Fourest *et al,* 1994). The algae species *Scenedesmus*, *Selenastrum* and *Chlorella* accumulated copper, lead and chromium from initial solutions of 100 mg/l with a 67 to 98 % efficiency (Brady *et al*, 1994c). *R. arrhizus* maximum biosorption of uranium, lead, lanthanum and zinc was 0.80, 0.50, 0.37 and 0.28 mmol/g, respectively (Tobin *et al*, 1990). Alkali treated *Aspergillus niger* sequestered silver ions at a rate of 0.92 mmol/g (Akthar *et al*, 1995) . The biosorption of thorium and uranium by inactive cells of *Rhizopus arrhizus* was 0.71 mmol/g and by pure cell wall components 0.88 mmol/g (Tsezos and Volesky, 1982a and b).

1.5 Mechanisms of metal removal

Due to the complexity of biosorbents and the numerous biosorption possibilities the mechanisms of metal binding and uptake are still relatively poorly understood (Volesky, 1992). Removal of metals from aqueous solutions by microorganisms generally comprises two phases. An initial rapid phase of passive retention of metal ions at the cell surfaces and a slower second phase by an apparent metabolism dependent intracellular uptake (Norris and Kelly, 1979; Garnham *et al*, 1992b).

1.5.1 Metabolism-independent metal biosorption

Non-active uptake mechanisms are varied and any one or a combination may be operational, including ion exchange, adsorption, chelation and microprecipitation (Volesky, 1987). Sorption of metals on the cell wall is mainly due to:

1. adsorption; which is a process in which the metal binds to unoccupied sites with no changes to the site, and
2. ion exchange; where the metal displaces another ion in the sorption process

(Crist *et al*, 1994).

Metabolism independent adsorption is rapid and results from a net electronegative charge of the cell wall interacting strongly with electropositive metallic ions (Beveridge, 1986). The adsorptive capacity of cell walls is determined by the structural organisation of the entire protein-carbohydrate complex and the availability of the functional groups to the metal (Davidova and Kasparova, 1992b; Brady and Duncan, 1994d). The anionic ligands of phosphoryl, carbonyl, sulphydryl and hydroxyl groups present on the cell wall are the functional groups active in metal immobilisation (Volesky, 1987). Tobin *et al* (1984) reported the amount of uptake by *R. arrhizus* of a variety of different cations was directly related to the ionic radii, with an increase in uptake corresponding to larger ionic radii. Differences in binding capacities occurring between species may reflect differences in cell wall composition (Gadd, 1986).

Ion-exchange can be expressed by the equilibrium equation: $M^{2+} + (AX_2) \rightleftharpoons A^{2+} + (MX_2)$, where X^- is an anionic binding site, M^{2+} a metal cation with a strong binding affinity and A^{2+} a weaker binding metal (Crist *et al*, 1994). Kuyucak and Volesky (1989a) found the biosorption mechanism of cobalt by *Ascophyllum nodosum* was predominantly by an ion exchange between cobalt and calcium ions. This competition for sorption sites was also demonstrated on yeast cell walls by Davidova and Kasparova (1993a) for a range of radio-labelled cations. Crist *et al* (1994) reported that the sorption of lead and cadmium on the alga *Rhizoclonium* caused the release of sodium, calcium and magnesium. Sorbed metals were removed by precipitation as sulphides, hydroxides or as EDTA complexes in ion exchange processes. The advantage of reversibly of ion exchange is that it allows for the reuse of the biosorbent. Tobin *et al* (1990), by chemical modification of the cell wall, identified phosphate and carboxyl as important moieties involved in the binding of lanthanum, lead, zinc and uranium by *Rhizopus* biomass. Scatchard plots reflected multiple, nonequivalent metal binding sites. They proposed a complexation uptake mechanism which was fully reversible by addition of complexing agents, competing co-ions or pH reduction, suggesting that ion exchange/complexation was the primary uptake

mechanism. Blocking of amino, carboxyl and hydroxyl groups of isolated *S. cerevisiae* cell walls by chemical modification decreased copper removal (Brady and Duncan, 1994d). This investigation showed the importance of these protein and carbohydrate associated ligands in the accumulation of metals.

The adsorption and immobilisation of metal may itself be a multi-phase process as is proposed for the mechanism of uranium biosorption by *R. arrhizus*. Uranium is initially complexed to the cell wall chitin. It then coordinates to the amino nitrogen of the chitin and is retained within the cell wall. A temporary equilibrium occurs followed by hydrolysis of the uranium-chitin complex and deposition of the hydrolysis product on the cell wall. The relieved metal binding sites accumulate further uranium until the process is inhibited by the hydrolysis product. These processes were proposed to be interdependent (Tsezos and Volesky, 1982a). Two processes participated in the biosorption of thorium by *R. arrhizus*. The first coordinated thorium to the amine nitrogen of the cell chitin network. The second process, which dominated overall biosorptive uptake, involved adsorption of thorium by external sections of the fungal cell wall (Tsezos and Volesky, 1982b).

In a subsequent investigation using *Ganoderma lucidum* as the biosorbent, Muraleedharan and Venkobachar (1990) proposed that the cell wall matrix opens up with copper contact. Using electron paramagnetic resonance they consistantly found a free radical trapped within the cell wall matrix. The detected signal strength of the free radical declined drastically after copper sorption. The free radical did not interact with the metal, was not relieved when metal was desorbed and neither did it interact with the dilute acid used for desorption. By opening of the matrix originally embedding the free radical on copper sorption, the free radical was allowed to attenuate. The exposed matrix could then interact freely with metal resulting in high removal rates.

Garnham *et al* (1992c) reported on the biosorption of technetium to different algal species. The predominant ionic species under aerobic conditions is the pertechnetate ion

(TcO_4^-). Accumulation consisted of a single rapid energy independent phase which was pH and concentration dependant. Uptake was relatively low compared to the cationic metals which is due to the overall negative charge of the algal cell wall. Biosorption of anionic metal species have been described as an electrostatic binding to protonated amine and imidazole groups.

1.5.2 Metabolism-dependent metal bioaccumulation

Metabolism-dependent removal involves the internalization of metal ions. Fuhrmann and Rothstein (1968) found the divalent cations nickel, cobalt and zinc bound reversibly to surface sites and in addition they were transported into a non-exchangeable pool in the yeast. Norris and Kelly (1977) found the uptake of cations by *S. cerevisiae* a progressive, slow process in which relatively large amounts of metals accumulated intracellularly and associated with this metal uptake was a potassium ion release. Physiological-cation efflux from cells post metal exposure has been observed by several researchers. Brady and Duncan (1994e) observed a rapid loss of approximately 70 % potassium from *S. cerevisiae* cells during copper accumulation, while 60 % of intracellular magnesium was lost. The heavy metals caused lesions of the vacuole membranes due to either a physical or metabolic interaction. Ohsumi *et al* reported a K^+ efflux from *S. cerevisiae* cells, when incubated with copper chloride ions, was caused by a selective change in the permeability barrier of the plasma membrane of intact cells with no effect on the vacuolar membrane. Norris and Kelly (1977) demonstrated a potassium loss of 2 K^+ for every cobalt ion accumulated. Cabral (1989) found the rate and amount of potassium release from *Pseudomonas syringae* cells was dependent on the initial copper concentration and was related to a progressive decrease in the cell viability. The release of K^+ was accompanied by a leakage of other cell metabolites including inorganic phosphates and nucleotides. The loss of anionic metabolites may play a role in the maintenance of the electric charge balance in the cell cytoplasm (Cabral, 1990).

Belde *et al* (1988) found cadmium provoked an efflux of K^+ from yeast cells which was greater in metabolizing cells than non-metabolizing cells, indicating cadmium had to enter

the cells before exerting its action. K^+ is primarily required by microbial cells as an osmotic regulator, a regulator of internal pH and as an enzyme activator. Gadd and Mowell (1983) found no simple relationship between amounts of cadmium taken up by *S. cerevisiae* and potassium release. The potassium release was due to membrane disruption caused by cadmium binding to the organic ligands. Avery *et al* (1992) showed the uptake of caesium caused a 71 % loss of intracellular K^+ from respiring *Chlorella emersonii* which led to a subsequent inhibition of growth. They demonstrated the inhibition of growth was a result of loss of K^+ below a threshold level required to support cell division and not as a result of the accumulated caesium. This concurs with earlier reported results of Kessels *et al* (1985) who showed that the cadmium induced potassium release was decreased by calcium which decreased the toxic effect of cadmium on yeast growth. Gadd and Mowell (1983) suggested the mechanism of cadmium toxicity was membrane disruption which resulted in K^+ and Mg^{2+} being released from the *S. cerevisiae* cells.

The cell membrane is an important site of interaction with metal ions as it is the site of different ion transport channels and it serves as a selective barrier controlling the influx and efflux of metal ions. The need for regulation of cellular uptake of metals is to ensure physiological processes are not inhibited. Transport systems have varying specificity for metals and rely on the electrochemical proton gradient across the cell membrane generated by H^+-ATPases of the membrane. The gradient of K^+, an 'energetical partner' of H^+, is used to increase the energy of the H^+ gradient (Okorokov, 1985). A primary event in cation uptake is the efflux of H^+ ions, mediated by the ATP driven H^+ pump. Increased cell ATP and stimulation of membrane ATPase activity increased cation uptake, with evidence suggesting the electrical potential, established by the plasma membrane ATPase, provides the energy for the transport of divalent cations into the cell (Borst-Pauwels, 1981). Both the chemical component (pH gradient) and the electrical component (membrane potential) can drive cation solutes across the membrane (Gadd, 1990).

Metal ion transport into cells can occur via transport carriers specific for physiological

essential ions such as magnesium and calcium. For example, calcium influx into yeast cells is through a calcium channel in the plasma membrane (Eilam and Chernichovsky, 1987). The plasma membrane functions as a barrier for Ca^{2+} influx or exchange under conditions where there is no metabolic energy, via a mechanism which involves the closure of Ca^{2+} channels at a low membrane potential. The calcium channel in the plasma membrane may be opened by an increase in the membrane potential or by the addition of metabolic substrates. Eilam and Chernichovsky (1987) determined that after 20 s of uptake, calcium was located in the cytoplasmic pool and after 3 min of exposure most of the cellular calcium was concentrated in the vacuole.

Metals may also be transported into microorganisms by non-specific transport of metal complexation to substrates which serve as carrier molecules through a transport system specific for the particular substrate. Chmielowski and Klapcińska (1986) demonstrated a biphasic uptake of germanium by *Pseudomonas putida* in the presence of a catechol substrate. The authors proposed that the catechol spontaneously bound germanium into a complex and the germanium could then be transported into the cells by the inducible catechol transport system.

Metal removal may also mediated by the release of cell components under stressful environmental conditions. *Pseudomonas syringae* cells suspended in a Na^{+}-piperazinediethanesulphate buffer and starved of external nutrients responded by metabolizing intracellular substrates such as RNA (Cabral, 1992). There was a subsequent release of UV absorbing materials, free and purine-bound ribose and inorganic phosphate. The products of endogenous metabolism had strong metal binding properties. Copper incubation with the filtrate from the starved cells led to considerable amounts of the copper being bound by the molecules in the filtrate. The release of these metal binding substances had no connection to any mechanism of metal detoxification.

Microorganisms can transform metal species by oxidation, reduction and methylation (Wakatsuki, 1995; White *et al*, 1995) These microbial transformations may be associated

with a decrease in solubility and toxicity. Lovley (1992) outlined the potential of metal reduction as a useful mechanism of bioremediation of contaminated environments. As an example, soluble lead (II) was reduced to insoluble lead (0) by aerobically grown *Pseudomonas maltophilia*. The reduced lead accumulated as a gray-black colloid which aggregated and settled out of solution. *Desulfovibrio desulfuricans* has been used to enzymatically reduce uranium from mine drainage waters which then precipitated as a uranyl carbonate complex. The process gave a high removal per cell and yielded a relative pure, compact uranium precipitate (Lovley and Phillips, 1992). Chromium (VI)-reducing bacteria have been used for the transformation of chromium (VI) to the less water soluble chromium (III). The system required a carbon source and was inhibited by co-contaminating metals which may render biological treatment of industrial effluents less efficient. Chromium (VI) toxicity may also lead to cell inactivation and the loss of chromium (VI) reduction (Losi *et al*, 1994; Wang and Shen; 1995). Mercury (II) is actively transported through the cell membrane and reduced to mercury (0) by the enzyme mercuric reductase. Elemental mercury diffuses from the cell and is lost to the aqueous phase by volatization (Hutchins *et al*, 1986).

S. cerevisiae is able to utilize ferric chelates as an iron source by enzymatically reducing the ferric iron to the ferrous form, which is subsequently internalized by the cells (Anderson *et al*, 1992). The enzyme ferric reductase in the plasma membrane of *S. cerevisiae* is activated in responsive to high external iron levels. The absorption and cellular distribution of iron cations in brewers yeast was found to be dependent on physiological status and growth stage of the cells and the iron was extensively located in the mannoprotein of the cell wall (Mochaba *et al*, 1996a). Microorganisms also enzymatically reduce other metals such as copper, silver and gold. The redox changes affect the solubility of metals and could be applied in bioremediation strategies.

1.6 Metal resistance

Microorganisms are the initial living systems exposed to the hostile environments created by metals. The ability to resist high concentrations of toxic metals has arisen in distinct

patterns. Firstly through an evolution under extreme natural conditions and secondly through a more recent acquired transferred resistance to a polluted environment through the acquisition of extrachromosomal DNA molecules (Wood and Wang, 1983). Basic measurements of the development of resistance to a toxic metal are: (1) the concentration of the metal must be increased to obtain the same deleterious effect; and (b) the same concentration of the metal causes a smaller effect than before (Ashida, 1965).

Toxicity results from nonspecific interactions of metal ions and cellular components causing the disruption of normal metabolism of essential metals. The frequency of manifestation of bacterial resistance to specific toxic metals appears to correlate to increasing loads of metals in the environment and for this reason they can be used as biological monitors of environmental contamination (Gelmi *et al*, 1994). The most common techniques for acquiring metal tolerant microorganisms include isolation from contaminated sites (Dressler *et al*, 1991; Kaplan *et al*, 1995), ultra-violet radiation and selection of resistant mutants (Pümpel *et al*, 1995); and genetic manipulations (Mergeay, 1991; Mergeay and Springael, 1997).

Belde *et al* (1988) developed a cadmium resistant *S. cerevisiae* strain by growing the cells in increasing amounts of $CdCl_2$, starting at 0.001 mM and doubling this concentration at each transfer to new medium until 2 mM $CdCl_2$ was reached. Uptake of cadmium into cadmium sensitive yeast cells was four times higher than in cadmium resistant cells (Belde *et al*, 1988). This increased bioaccumulation was ascribed to permeabilization of the cells. Brady *et al* (1994f) developed a metal tolerant *S. cerevisiae* strain which could survive toxic copper levels of 10 mmol/l, however its metal removal capabilities remained less than the parent strain.

In a bioremediation process metal resistance should always be associated with an increase in metal removal, such as a nickel resistant *Neurospora crassa* mutant which sequestered up to 90 % of a 120 ppm nickel solution, representing a 60 % improvement over the parent strain (Kumar *et al*, 1992). Wong and So (1993) isolated a copper resistant strain

of *Pseudomonas putida* which accumulated the metal at levels as high as 6.5 % of it's dry weight.

Microorganisms adapt by developing unique mechanisms which allow them to function even in the presence of high levels of these toxic agents (Lundgren *et al,* 1986). These include the modification of specific ion transport systems and the production of reductases which convert the metals to non toxic or insoluble forms. The cells may also produce extracellular chelating compounds. The metals may be immobilized into vacuoles normally used for storage of physiologically essential metals.

Mutants of *S. cerevisiae* carrying a methylgloxal resistance gene showed resistance to divalent cations cadmium, copper and nickel. This multiple resistance was dependent on the excretion of large amounts of glutathione into the medium. The metals have an affinity for the thiol group of glutathione and form extracellular metal-glutathione complexes (Joho *et al,* 1995). Survival after exposure to cadmium levels of 100mg/l was enhanced for *Klebsiella* species capable of producing exopolysaccharides, which sequestered the metal externally and contributed to the organisms defences (Scott and Palmer, 1990). The resistance by the microorganism was thus achieved by preventing the metals from entering the cells. *S. cerevisiae* resistance to copper may also be mediated by excess production of the copper-binding protein metallothionein. Yeast metallothionein is a polypeptide of 61 amino acids which complex the metal as copper (I) to cysteinyl thiolates (Mehra and Winge, 1991). The metallothioneins act as intracellular traps which prevent metal concentrations in the cytosol reaching toxic levels. This serves as a temporary measure preceding expulsion of the metals from the cell by vacuoles (Wood and Wang, 1983).

White and Gadd (1986) reported that the repeated culture of the yeast *S. cerevisiae* at elevated concentrations of cobalt led to increased cobalt accumulation in the vacuoles. Manganese resistant mutants of *S. cerevisiae* were selected by Bianchi *et al* (1981). The resistance was due to mutations on a single nuclear gene (MNR1). The mutants could be

grown in manganese concentrations of 200 mM which was completely cytostatic for wild type cells. Insoluble manganese in the mutants was 70 fold higher, at a 200 mM initial concentration, than that in sensitive cells. This suggested a mechanism for precipitation and a hypothesis of an enhanced sequestering capacity in the resistant strains was proposed. Intracellular homeostasis in the cytosol is regulated by a specific transport system using the electrochemical gradient (Joho *et al*, 1995). The vacuolar H^+-ATPase of nickel resistant *S. cerevisiae* cells was less sensitive to the metal than the parent strain and enhanced sequestration of nickel from the cytosol into the vascular compartment occurred in the resistant cells.

Several genes associated with specific metal resistance mechanisms have been isolated, such as the COT1 and ZRC1 genes of *S. cerevisiae* (Conklin *et al*, 1994). The COT1 gene encodes a 48 kDa protein which contains several potential amino acid binding sites. The expression of COT1 confers cobalt resistance by increasing sequestration and compartmentalization of the ion in the mitochondria (Conklin *et al*, 1992). The ZRC1 gene product is a protein of 442 amino acid residues which confers increased resistance to elevated zinc concentrations. Multiple copies of the gene enabled yeast cells to grow in the presence of 40 mM zinc. Kamizono *et al* (1989) proposed that the resistance mechanism of the gene product was possibly due to it being a membrane protein actively involved in pumping zinc out of the cell. *S. cerevisiae* metallothioneins are encoded by the CUP1 gene on chromosome VIII. Resistant strains contain multiple copies of the gene which are induced in the presence of copper. Fogel and Welch (1982) proposed resistance mediated by a gene amplification mechanism. Over expression of CUP1 in recombinant yeast, containing multiple copies of the gene on plasmids, increased the critical copper resistance level 4 fold (Had *et al*, 1992). The CUP1 gene plays a common role in the resistance of yeasts to more than one species of metal. Copper (I) and silver (I) associated with the protein at a stoichiometry of 8 ions per molecule; zinc (II) and cadmium (II) association was at a stoichiometry of 4 ions per molecule (Winge *et al*, 1985, Tohoyama *et al*, 1992).

The survival response and increased metal removal due to specific metal resistance mechanisms is of importance to a biohydrometallurgist as a means of maximizing the exploitation of the biosorbent and optimizing metal bioccumulation from contaminated waters.

1.7 Nutritional requirements and physiological status of the biosorbent

The growth media of the cells can have a considerable impact on their potential to accumulate metal by energy-dependent and -independent mechanisms (Ercole *et al,* 1995). Commercial baker's yeast metal accumulation was enhanced between 5-20 fold in the presence of glucose (Fuhrmann and Rothstein, 1968) *S. cerevisiae* cells grown in media supplemented with additional glucose, ammonium, phosphate and cysteine led to different functional groups on the corresponding cell walls. Cysteine inserts S- and N-ligands, glucose C-ligands, ammonium N-ligands and phosphate P-ligands which effect metal binding. Zinc bound best to cultures from phosphate enriched media, provoking zinc selectivity. Cells grown in cysteine supplemented media had the highest general biosorption capacity (Engl and Kunz, 1995). Mochaba *et al* (1996b) found the physiological condition of yeast drastically affected the extent of metal uptake by brewers yeast with less vital cells being ineffective at concentrating metals. Bacterial cells starved of external nutrients released strong metal binding molecules (Cabral, 1992). The uptake of cadmium in two strains of *Saccharomyces* was investigated in the presence and absence of glucose by Rösick *et al* (1986). In the presence of glucose cadmium uptake increased approximately 5 fold compared to uptake by cells in the absence of glucose. The authors concluded that this was due to an energy-dependent transport system.

1.8 Metal binding kinetics-characterization of metal uptake

The removal of metals by microorganisms are often characterized using Michaelis-Menten kinetics, the Langmuir model, the Freundlich model, and others. Although various models have been developed to characterize metal removal, the Michaelis-Menten model was preferred in this study as it has specific benefits for biological systems. Initially developed for the study of enzyme catalysed reactions, the model is easily adapted to metal

bioaccumulation. It allows different parameters affecting binding to be taken into account with the influence of secondary compounds or metals being characterized as inhibitors or as multiple substrates. The removal of metals is often complex and more than one mechanism may be in operation at any one time, which can be observed using the above model. The Michaelis-Menten kinetic characterization of metal removal is accepted and used in the biological sciences for this reason (Failla *et al*, 1976; Borbolla and Peña, 1980; Garnham *et al*, 1992b; Borst-Pauwels, 1993; Gadd and Lawrence, 1996).

1.8.1 Adapted Michaelis-Menten model

The adapted model follows the simplified reaction:

$$[Y] + [M] \rightleftharpoons [YM]$$

Where [Y] is the free cell concentration in solution,

[M] is the free metal concentration,

[YM] is the amount metal bound per gram of cells.

The scheme is used to derive a mathematical expression describing the relationship between initial velocity of uptake and the metal concentration:

$$q = (B_{max} \times [M])/(Kd + [M]) \qquad (1)$$

Where $q = [YM]$,

$[B_{max}] = [Y] + [YM]$ and represents the amount of maximum uptake per gram of cells,

Kd is the dissociation constant of the metal yeast complex at equilibrium and can be determined graphically as the [M] yielding $q = B_{max}/2$. The reciprocal of Kd is Ka, thus the greater the affinity of binding, the smaller the Kd concentration.

The assumptions of this model are:

1. an equilibrium between biosorbent, metal and biosorbent metal complex would be almost immediately set up and maintained;
2. metal is generally present in greater concentrations than binding sites (Palmer, 1991).

Linear forms of the Michaelis-Menten equation are accurate means used to determine Kd and B_{max}. Linear plots often used are:

Lineweaver-Burk	-	$1/q = Kd/(B_{max} \times [M]) + 1/B_{max}$	(2)
Hanes-Woolf	-	$[M]/q = [M]/B_{max} + Kd/B_{max}$	(3)
Eadie-Hofstee	-	$q = (-Kd \times q)/[M] + B_{max}$	(4)
Scatchard plots	-	$q/[M] = (Kd \times B_{max}) - (Kd \times [AM])$	(5)

Characterisation of data using the Scatchard transformation of binding isotherms possibly represents the most suitable model to determine Kd and B_{max}. The extent of binding is expressed by a ratio of bound to free metal obtained by a linear transformation (equation 5) of the saturation isotherm. A plot of q/[M] versus [AM] results in a linear plot if the assumptions of the model are correct. An extrapolation of the X intercept corresponds to the B_{max} and the slope represents -Kd. The Scatchard model assumes specific binding, forming a monolayer on a surface of repeating, energetically equivalent, surface functional groups. These assumptions are not strictly correct for biological cell surfaces and the data generated from the model only permits a generalization of metal biosorption characteristics (Cho *et al*, 1994). However these plots can be used to interpret multiple, nonequivalent binding sites depending on the curvature of the transformed plot (Tobin *et al*, 1990).

1.8.2 The Langmuir model

The Langmuir model used to determine sorption parameters has the hyperbolic equation:

$$q = (Q \times C)/(Kd + C)$$

Where q = metal uptake,

C = residual concentration,

Q = maximum sorption capacity,

Kd = dissociation constant.

The Langmuir sorption isotherm model is a monomolecular model adapted to equilibrated and saturable chemical sorption at unique and specific sites. It considers the metal, the biosorbent and the complex formed. The basic assumptions on which the model is based

are:

1. Metal ions are chemically adsorbed at a fixed number of well-defined sites;
2. Each site can hold one sorbate ion;
3. Each site is energetically equivalent;
4. There is no interaction between ions adsorbed on neighbouring sites (Ruthven 1984, cited from Fourest and Roux, 1992).

From section 1.5 it is clear that metal removal involves many different mechanisms. As with the Scatchard plot, these basic assumptions may not always be correct for metal removal by biosorbents (Crist *et al*, 1994).

The model uses the equilibrium concentration and not the initial concentration. It is argued that as the sorbent is only exposed to the initial concentration for a very short time it is fundamentally more correct to use the equilibrium at steady state (de Carvalho *et al*, 1995). The counter argument is that when using laboratory data to predict the efficiency of a particular bioremediation protocol for industrial waste water, the initial information supplied by a site manager would be the initial metal concentrations which are decisive in predicting the system's potential.

The future development of metal bioremediation technology is dependent on the successful application to industrial waste waters. The experimental methodology and data analysis should be designed with application as the ultimate objective. Investigators often describe complex, multi-faceted binding mechanisms by a particular organism, then characterize the metal uptake using a one dimensional model and if a linear plot is obtained from data transformation, the metal uptake is proposed to follow the adsorption model (Tsezos and Volesky, 1982a; Volesky *et al*, 1993). This may be misleading and a more clear method of data interpretation may be required considering the interdisciplinary nature of the research. A possible solution, to allow simple comparisons of data between laboratories, would be to use a set of well defined parameters with data reported as the absolute values obtained, and not as a theoretical modelled value. The

analysis of data would need to include the incubation time, initial metal and biomass concentration, residual metal concentration after incubation and pH. Most important would be the optimum removal efficiency of the biosorbent at a defined initial metal concentrations per gram of biosorbent.

1.9 Metal recovery from biosorbents

The potential application of a biosorbent depends not only on its uptake capacity, but also on the ease of recovery of the bound metal. The ability to regenerate and reuse the biosorbing material has important financial implications for the practical use of the technology (Kapoor and Viraraghavan, 1995). Increasing demands and diminishing resources of valuable metals necessitates the recovery of these metals. As microorganisms form the basis of the food chain they are potentially capable of transferring toxic metals to higher trophic levels and, therefore, from an environmental perspective, bioaccumulated metals need to be recovered and removed from the biosphere (Hart and Scaife, 1977). An efficient desorption process needs to be developed to safely reclaim bioaccumulated metals and to allow reuse of the biomass. A wide range of potential eluants has been investigated with success being largely dependent on the type of biosorbent, the metal and the mechanism of accumulation employed by the microorganism.

Kuyucak and Volesky (1989b) employed calcium chloride (0.05 M) in HCl at pH 2-3 as an eluant for cobalt from metal-laden *Ascophyllum nodosum* and recovered 96 % of the bound metal over a 2 hr period. Up to 75 % of bioaccumulated nickel and zinc was recovered from a sulphur bacteria of *Thiothrix* spp using 5 mM $CaCl_2$, the primary mechanism of desorption being ion exchange (Shuttleworth and Unz, 1993). Copper bound to *Chlorella vulgaris* and *Scenedesmus quadricauda* was desorbed with 0.05 M sodium acetate at pH 2 (Harris and Ramelow, 1990). Cobalt was desorbed from immobilized *C. salina* by dilute mineral acids, however, prolonged exposure killed the cells preventing their reuse (Garnham *et al*, 1992a). Deposited cadmium was desorbed from *A. nodosum* through 5 cycles using 0.1 to 0.5 M HCl with no decrease in metal uptake capacity (Holan *et al*, 1993). Treen-Sears *et al* (1984) demonstrated the repeated

adsorption-desorption capabilities of *R. arrhizus* over eight cycles for uranium using nitric and sulphuric acid eluents. Tsezos (1984) found uranium removal from *R. arrhizus* was best using sodium bicarbonate because of near complete recovery, high concentration factors and least damage to the cells. Sodium carbonate was used as a stripping agent for metal recovery by Nakajima *et al* (1982). A successful desorption protocol would improve the economic viability of a bioremediation process which is dependent on the metal, and the manner in which it has been sequestered.

1.10 Industrial effluents and application of bioremediation

The application of a biosorbent to a bioremediation system is dependent on different factors which include the effectiveness of metal removal, availability of the biosorbent and its cost, regeneration of the biosorbent and the ease of use of the technology. The cost of a fungal biomass compared to activated carbon and ion exchange resins indicates that the biomass would be competitive (Kapoor and Viraraghavan, 1995). The cost of ion exchange resins are approximated at R 60 to R 135 per kg, activated carbon between R 10 and R 25 per kg, biomass cultured specifically for bioremediation between R 5 and R 23 per kg, and biomass obtained as a waste-product from industrial fermentation would be dependent on transport and drying costs. The technology may convert the waste-product to a by-product which may add to the cost.

The viability of the biosorbing cells is a criteria which requires consideration when utilised for bioremediation. While both living and dead cells accumulate metals, different mechanisms are utilised. Depending on the metal accumulated, varying degrees of toxicity toward living cells are exhibited. Toxicity problems are not a consideration when using a non-viable biomass or derived constituents thereof. The removal of metal by dead cell material may sometimes increase as it loses active defence mechanisms to toxicity, which decrease removal of the heavy metals (Volesky, 1992). Non-viable biomass functioning as a physico-chemical process would not require the addition of substrates and would be a simple and more cost effective system. Living cells may provide an advantage because of the variety of accumulation mechanisms and their ease of manipulation (Gadd, 1990).

The mechanism of removal is also of importance when considering application of a biosorbent and it's reusability. Surface bound metals can easily be recovered with mild chemical elution, however, intracellular metal may require cell disruptive methods for recovery, which would prevent reuse of the biomass for further applications.

Metal wastes from industry represent a substantial loss of non-renewable resources which are causing considerable toxic and heavy metal contamination of the environment. Recovery of these metals in a concentrated and manageable form is the challenge facing the environmentally concerned metallurgist. Present treatment techniques of metal contaminated industrial waste waters have the drawback of high operational costs and the disposal of resulting metal sludges remains labourious. The potential of the bioremediation technology is considerable, particularly the prospect of providing an environmentally sound and cost effective removal process, with the additional incentive of the recovery of valuable metals. In the design of a wastewater treatment system, the operation and the characteristics of the effluent are interdependent considerations requiring thorough investigation (Moo-Young and Chisti, 1994). A guideline to a remediation process is shown in Figure 1.1.

Design of bioreactors are based on biosorbent-effluent contact, allowing sufficient mixing for maximum metal removal. Batch reactors, in which effluent and biosorbent are mixed together in large tanks and stirred, represent the simplest operation. The metal-laden biosorbent is then separated from the liquid phase, the metal desorbed and the biosorbent reused. The liquid/solid separation is the most challenging part of the operation.

An alternative is the packed bed system, which alleviates the requirement of additional liquid/solid separation procedures. Advantages of packed bed bioreactors are ease of biomass regeneration and continuous operation (Tobin *et al*, 1994). Disadvantages include relatively low effluent treatment volumes, slow flux rates and clogging. White and Gadd (1990) reported stirred and static bed bioreactors did not give satisfactory removal because of poor mixing.

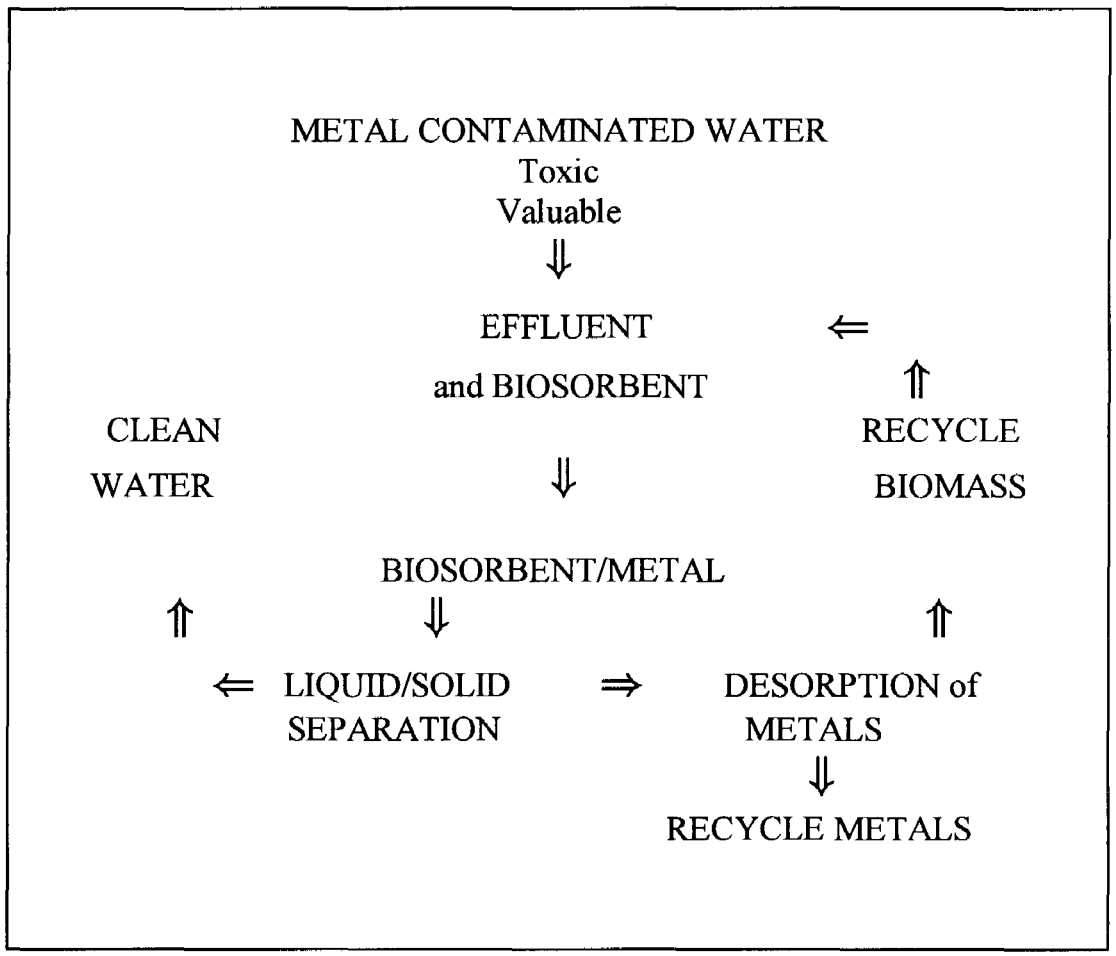

Figure 1.1 : A general scheme of metal remediation and recovery cycles from aqueous solutions by microbial biomass. Adapted from Gadd (1990) and Summers (1992).

An air-lift reactor was found to promote circulation and increased contact between the biomass and the metal. A continuous biosorption air-flotation operation was reported for cadmium removal by suspended biomass by Matis *et al* (1996). Air-flotation was used for solid/liquid separation of metal laden biomass from solution, with metal removal and biomass recovery of above 95 % being achieved. The batch reactor flotation separation yielded a metal loaded biomass and a clean water underflow stream (Matis *et al*, 1994).

Reactor design is of importance when considering the scaling-up of a bioremediation protocol for application to the high volume industrial waste waters. The efficiency of metal uptake is further influenced by particle size, shape and density of the biomass;

density, viscosity and flow rates of the metal bearing reaction solution (Shumate *et al*, 1980).

A system of microbial mats constructed of cyanobacteria and inoculums from metal contaminated sites has been investigated by Bender *et al* (1995) for practical applications for high volume effluents. The advantages of this system are the low cost, tolerance to high metal concentrations and the potential to provide a large surface area and a rapid flow of contaminated water.

The removal of microbial contamination is an important factor in ensuring the safety of public water supplies (Pieterse, 1989). Thus the choice of biosorbent, it's viability, solid/liquid separation and the destination of the water are important considerations. The choice of biosorbent is of less consequence if the recycled water is reused on site in the industrial process, however, the biosorbent may itself present a health hazard if discharged into the environment. A second example of the negative consequence of a particular microorganism was noted by Osborn (1989). The high concentrations of sulphates and organic matter often present in recycled water provide stable conditions for the growth of *Desulfovibrio desulfuricans* which proliferate in ageing reticulation pipes, causing perforation. This can lead to substantial water loss and the cost of replacing water reticulation pipes is high.

Reported transfer of the technology from the laboratory to the industrial site has been limited. Problems relating to metal bioremediation are reviewed by Summers (1992). The most problematic aspect of applying the technology is the complexity of most effluents. Co-contaminating metals and other compounds can severely decrease remediation efficiency and at the very least make the process highly erratic and unpredictable The target metals cannot be transformed to harmless products. They have to be recovered and preferably removed from the biosphere. Bioremediation of metals by metabolic-dependent mechanisms are limited to the environmental conditions and environments which inhibit cell physiology may not be suitable for application. Commercial applications

of bioremediation have been infrequent and remain the major challenge for the biohydrometallurgist.

A microbial agent for gold recovery from a jewellery manufacturer's wastewater was reported by Brierley (1987). The biosorbent recovered gold of up to 155 mg/g from an residual concentration of 885 mg/ℓ. However, bioremediation of dilute gold effluents with high cyanide concentrations only yielded 27 mg/g which had no advantage over activated carbon used in the industry (Brierley, 1987). Gale (1986) investigated heavy metal accumulation by algae in treatment ponds and receiving streams of mine wastewater. The uptake of metals by aquatic vegetation was monitored and high levels of lead, zinc, copper and manganese were detected. Although this study was reported as an example of natural environmental protection, the metals were not recovered and remained a risk in the biosphere. Velea *et al* (1995) reported nickel and copper removal from industrial wastewaters by strains of *Aspergillus* and *Penicillium*. The effluent metal concentrations were high and the removal efficiency of copper and nickel by *Aspergillus* was 41% and 45 % respectively and by *Penicillium* only 22% and 32 %.

1.11 *Saccharomyces cerevisiae* as a biosorbent

The *Saccharomyces sp* is one of the best studied and commercially exploited of all microorganisms (Stewart and Russell, 1985). *S. cerevisiae* is a by-product of large scale fermentation and it's annual production is in the order of millions of tons, thus it has the potential to provide large quantities of cheap biomass (Blackwell *et al*, 1995).

Yeasts require a minimum number of inorganic ions in micro and millimolar concentrations for growth and fermentation. The role of the ions in yeast cells are two fold: (1) enzymatic functioning as part of the catalytic centre of an enzymes, as activators of enzyme activity and as physiological regulators. Zinc, cobalt, manganese and copper are common in catalytic centres; magnesium acts as an enzyme activator and potassium commonly functions in the role of a metal co-enzyme; and (2) a structural function. Potassium and magnesium have a role in neutralizing electrostatic interactions between

cellular anionic units of polyphosphates, DNA, RNA and proteins (Jones and Greenfield, 1984). Ca^{2+}, Mg^{2+} and Zn^{2+} are often complexed to membrane phospholipids and cell wall phosphomannans. The presence of cations in yeast is significant with respect to its exploitation as a metal biosorbent. Yeast cells require metals for normal metabolic processes and readily accumulate a range of metals. The second point relates to the delicate intracellular ionic balance, with imbalances being reflected as alterations in metabolic processes and growth characteristics. Exposure of yeast from malt fermentation to high levels of metals in a bioremediation system would possibly lead to cell death and further metal removal would be by metabolism independent mechanisms.

The cell wall and plasma membrane are the most important components of the yeast cell in metal removal from aqueous solutions. The cell wall maintains structure and rigidity and is permeable to solutes smaller than 600 Dalton. It serves as the initial contact point and provides the negative functional groups for cation binding. The yeast cell wall consists of an intermesh of polysaccharide microfibrils in a matrix of various polysaccharides, proteins and lipids. The major constituent ($\geq$ 60 %) of the cell wall is the polysaccharide mixture of glucans, phosphomannans and chitin (Northcote and Horne, 1952; Bartnicki-Garcia and McMurrough, 1971; Phaff, 1971; Rose, 1993).

Chemical and enzymatic isolation of the cell wall components of *S. cerevisiae* indicated metal binding to each of the cell wall components. The isolated components accumulated greater quantities of cations than intact cells, with the outer mannan-protein layer being identified as more important for metal binding than the inner glucan-chitin layer (Brady *et al*, 1994g).

In contrast to bacteria with a single plasma membrane, *S. cerevisiae* cells contain a number of specialized membranes. Most important to metal transport are the outer membrane separating the cell components from the external medium and the vacuolar membrane which compartmentalizes different metabolically important compounds (Okorokov *et al*, 1980; Lichko, 1980). The plasma membrane is composed of a lipid

bilayer containing assymetrically located proteins. These proteins mediate the selective uptake and/or secretion of solutes. Transport of solutes through the membrane is mediated by primary and secondary transport proteins. Primary transport is the active process in which chemical energy is converted to electrochemical energy. An example is the hydrolysis of ATP by the plasma membrane ATPase enzyme, which generates an electrochemical gradient, which in turn drives the processes involved in solute transport (Gadd and Laurence, 1996). Secondary transport systems utilise the electrochemical gradients of primary transport systems and are divided into three categories: (1) a uniport system which is the transport of a single solute facilitated by a carrier protein, (2) a symport system which is the coupled movement of two or more solutes in the same direction and (3) an antiport system which refers to the coupled movement of solutes in opposite directions. The plasma membrane and its different transport systems is the most important yeast cell component for intracellular uptake of metals (van der Nest *et al*, 1995).

S. cerevisiae has the potential to fulfil most of the criteria required from a biosorbent for the application to a bioremediation process. These criteria include: (1) sufficiently high metal removal capacities; (2) a rapid metal uptake; (3) a low cost biomass; (4) a good resistance to chemicals and (5) an adequate size and strength to withstand the mechanical stresses of a bioremediation process. The size must also be sufficient to allow easy recovery of the biomass from solution post metal accumulation. Recovery of metal by desorption must be achieved with minimal cost, should be metal selective and preferably allow for reuse of the biosorbent (Tsezos *et al*, 1988). Contacting large volumes of metal bearing aqueous solutions with a natural biomass in process operations, can be problematic because of the difficulty of liquid/solid separation. A practical solution is immobilization of the biomass which confers an ideal size, mechanical strength, rigidity and porosity, and yields a biosorbent which can be recovered, stripped of metals, reactivated and reused (Brierley and Brierley, 1993).

A scanning electron micrograph of *S. cerevisiae* cells is shown in Figure 1.2. The large

cell number in a small volume would provide a large surface area for adsorption of metals in a bioremediation system. Due to the small size of the cells (2 to 5 μm) both free cell suspension and immobilized cell systems were investigated.

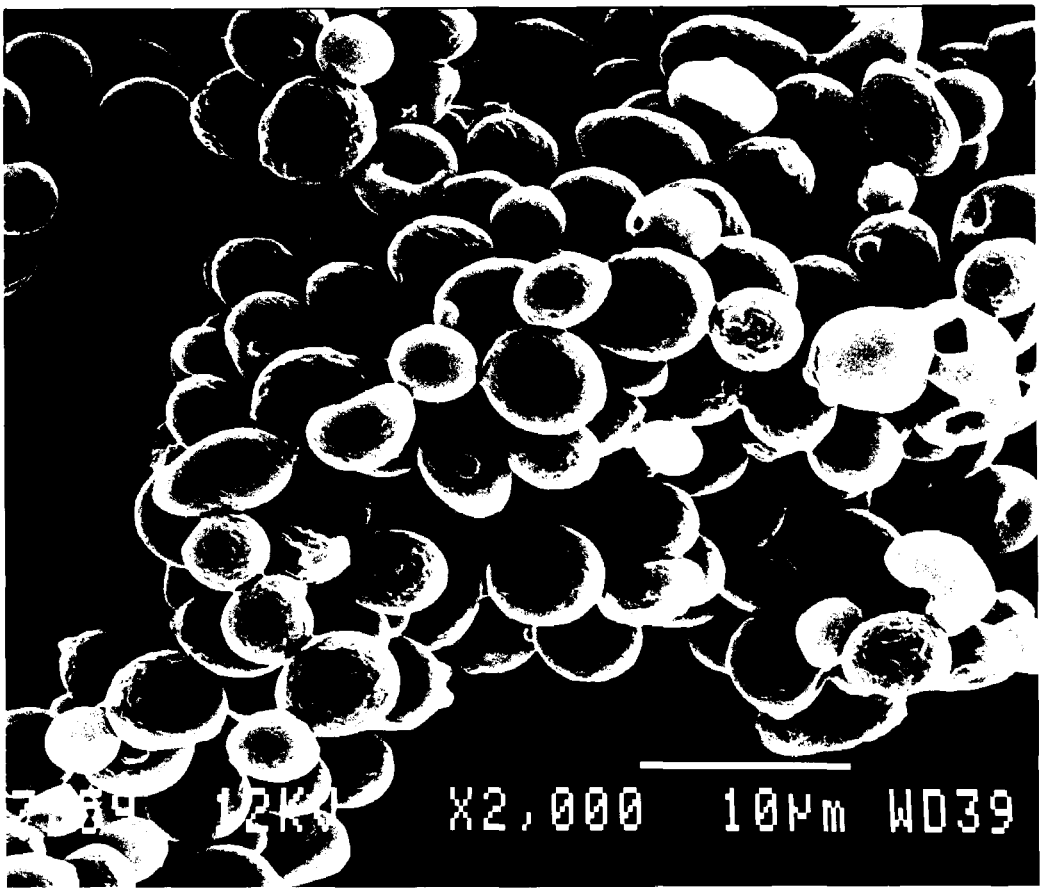

Figure 1.2: A scanning electron micrograph of *Saccharomyces cerevisiae* cells.

1.12 Research aims

This research project first undertook to assess the efficiency of using an immobilized baker's *S. cerevisiae* biomass for the bioremediation of metals. Initial studies focussed on the parameters affecting removal of metals from aqueous solutions in batch reactors. The kinetics involved in the bioaccumulation process were determined and maximum

binding capacities calculated.

The ability to recover biomass bound metals by mild chemical elution was then investigated. A range of potential eluting were investigated to establish a rapid, non-destructive protocol for metal recovery and concentration. The reusability of the biosorbent was assessed by repeated adsorption-desorption cycles. A semi-continuous laboratory scale bioremediation process using biosorption columns was then investigated and the metal uptake capacities and recovery potential assessed. Selective recovery of the metals from dual metal solutions was studied using biosorption columns linked in series. The potential use of the immobilized yeast biomass was investigated for the remediation of a metal contaminated mine effluent.

The use of a waste yeast which was obtained from the breweries post fermentation and its metal uptake capabilities was also investigated. The physiological status of the waste yeast was assessed before bioaccumulation to establish the relationship between initial viability of the yeast and metal accumulation. Energy dispersive X-ray analysis was used to determine the deposition sites of the metal on the cell. Dual metal studies investigated the competition for binding and uptake between the metals. The combined kinetic and X-ray analysis results were used to gain an insight into binding mechanisms and different metal affinities. The waste yeast was also investigated for its ability to remediate metal contaminated mine effluent.

CHAPTER II

THE REMOVAL OF METALS FROM AQUEOUS SOLUTIONS BY IMMOBILIZED *Saccharomyces cerevisiae*

2.1 INTRODUCTION

Immobilization of drugs, enzymes and whole cells have been investigated by various researchers for potential application in different biotechnological processes (Carenza and Veronese, 1994; Chibata *et al*, 1983; Venkatasubramanian and Veith, 1979). Bioparticles potentially have greatly improved mechanical properties for industrial processes after immobilization (Chamy *et al*, 1990). These properties include a particle size of 0.5 to 3 mm, a mechanical strength which resists abrasion and can withstand pressure and a resistance to chemicals (Tsezos *et al*, 1988). The immobilized cells should not relinquish metal accumulating characteristics such as high metal removal and recovery, low cost and selectivity as a result of the entrapment process (Volesky, 1987). The entrapment matrix should allow permeability to the metal species to be sequestered. Garnham *et al* (1992a and b) reported an improved accumulation of metals by the immobilized biomass compared to free living cells.

Advantages of immobilization to metal bioremediation processes are; improved strength and handling capacity, reduced likelihood of system blockages, and better regeneration characteristics (Tobin *et al*, 1993). These systems allow for better capability of biomass reuse, easy separation from the reaction mixture and high biomass loadings. The particle size can also be controlled (Subramanian *et al*, 1994). The diameter of the pellet particles should be small enough not to pose obstacles and blockage in a remediation process, and large enough to allow liquid/solid separation (Sumino *et al*, 1992). Singh *et al* (1992) reported that cells immobilized through suspension in a solution of sodium alginate and then pumped into $CaCl_2$, form hardened beads which have the advantage of greater cell

longevity. The strengthened biomass also has a greater potential for multi-step processes and is more adaptable to environmental disturbances.

Factors to be considered when using immobilized biomass are; the swelling rate, compressive strength and the respiratory activity of the pellets. Another important consideration is cell leakage from the binding matrix (Sumino *et al*, 1992). Tanaka *et al* (1989) described a decrease in cell loss by entrapment in alginate fibres with double gel layers. Cells were restricted to the inner layer while the outer layer prevented leakage. Diffusion hindrance can arise and cause an apparent decrease in reaction velocities which can potentially offset the advantages of the immobilized biosorbents (Westrin and Axelsson, 1991). Tsezos and Deutschmann (1990) reported a 30 % decrease in metal removal of an immobilized *R. arrhizus* biomass relative to the native biomass. The potential advantages and disadvantages of immobilization are particular to the immobilization technique and the biomass used, and require individual assessment to determine feasibility.

Conventional immobilization systems include; entrapment in a polyacrylamide gel lattice, gluteraldehyde crosslinking, polyurea, κ-carrageenan and sodium alginate encapsulation (Chibata, 1974; Chamy *et al,* 1990; Babu *et al*, 1974). More recently, more novel immobilization techniques have been investigated. Basnakova and Macaskie (1997) have suggested the priming of immobilized *Citrobacter* cells with a suitable metal phosphate. Microbial phosphatase hydrolysis of the organophosphate generates a phosphate ligand (HPO_4^{2-}) and the cation is removed as the phosphate biomineral ($MHPO_4$). This system has been used for nickel removal.

The efficiency of a metal bioremediation process is dependent on many factors including the biosorbents maximum binding capacity, metal affinity and bioavailability, and the composition of the effluent (Volesky, 1987; Blackburn, 1993). In this initial study the uptake kinetics of a range of metals by an immobilized bakers *S. cerevisiae* biomass were investigated. The parameters influencing the metal binding capabilities were investigated

and the potential advantages of the immobilized biosorbent explored. The parameters examined; were metal concentration, incubation time and pH. Optimum metal pH ranges and incubation times were determined and these parameters were used in the equilibrium binding studies. Metal uptake is represented as μmole of metal removed from solution per gram of *S. cerevisiae* (μmol/g). The metals initially chosen for this study were copper, cobalt, zinc, cadmium, nickel and chromium as they are known to occur at toxic levels in a wide range of industrial effluents. Gold was investigated because of its potential recovery value.

2.2 MATERIALS and METHODS

2.2.1 Materials

Commercial preparations of *S. cerevisiae* were obtained from Anchor Yeast Inc. (production strain 90 % viability). Copper, cobalt, cadmium chlorides were obtained from Merck, zinc chloride from BDH, chromium chloride from Reidel-de-Haen and nickel chloride from Saarchem. Gold standard was obtained from Saarchem. N,N,N',N'-tetramethylethylenediamine (TEMED), N,N' methylene-bis-acrylamide and polyacrylamide were purchased from Sigma. Ammonium persulphate, sodium chloride, sodium hydroxide and hydrochloric acid were supplied by Saarchem. HA 0.45 micron nylon filters were purchased from Micron Separations Inc. Merck supplied the 1,1,1,3,3,3-hexamethyl disilazane. Ultra-pure deionized water, purified by a milli-Q water system, was used to prepare solutions in all experiments.

Experimental design was such that the processes used in the laboratory could potentially be scaled-up and utilised in an industrial application. The metal solutions and reaction mixtures were not buffered by chemicals and the process maintained as simple as possible.

2.2.2 *S. cerevisiae* immobilization

Yeast immobilization as adapted from Brady and Duncan (1994a) was as follows: 3.75 g acrylamide monomer and 0.2 g N,N'-methylene-bis-acrylamide were dissolved in 12 mℓ

deionised water. *S. cerevisiae* (5 g) was washed with 10 mℓ water and then resuspended in 10 mℓ 0.9 % NaCl. The acrylamide solution and the cell suspension were mixed together with 1 ml TEMED and 2.5 mℓ ammonium persulphate. The reaction mixture was cooled and allowed to polymerize, after which it was passed through a 30-mesh sieve. The resultant biomass (± 50 g) was conditioned with washing in 0.1 M HCl, followed by a 0.05 M NaOH wash and a final rinse in water. The pH was adjusted to 5 and all excess water removed by filtration. A pH of 5 for the biomass was used as it was an optimum pH for bioaccumulation of a range of metals and was sufficiently low not to initiate metal precipitation.

2.2.3 Adsorption kinetic profiles

Immobilized biomass (1g) was weighed into 100 ml conical flasks. The metal solution (10 mℓ of 200μmol/ℓ at pH 5) was then added. For determination of the binding rates the contact times were 0.5, 1, 2, 3, 4, 5, 10, 20, 30 and 60 minutes with shaking at ambient temperature. Post incubation 5 mℓ of the reaction mixtures were filtered through 0.45 μm nylon filters under vacuum and the filtrate analysed for metal.

2.2.4 pH profiles

The pH of the metal solutions was adjusted using NaOH or HCl prior to incubation with the biosorbent. The pH range evaluated was from pH 2 to 6. Batch reactor incubation was as described for equilibrium profiles. The initial metal concentrations were 200 μmol/ℓ.

2.2.5 Equilibrium profiles

Investigations of metal removal by the immobilised biosorbent were conducted over the range of 50 to 2000 μmol/ℓ. Incubation was as above with shaking for 30 minutes. Post-contact, the mixtures were centrifuged at 500 g x 5 min, the supernatant was removed and analysed for metal. For kinetic characterization, the results were analysed using Michaelis-Menten binding isotherms. Kd and B_{max} values were determined using Scatchard and Hanes-Woolf transformation plots.

All the above experiments evaluated the removal the metal chlorides of Cu^{2+}, Zn^{2+}, Co^{2+}, Cd^{2+}, $Ni^{2+}$and Cr^{3+}. The gold solution investigated was in the form of Au^{+} and/or Au^{3+}. Appropriate batch reactor controls were run concurrently in all investigations. Controls containing equivalent quantities of polyacrylamide gel with no yeast were used to determine the extent of binding to the immobilizing gel. A second set of controls contained only the metal solution to determine the extent of extraneous metal removal throughout the process. The determinations were repeated 5 times per parameter evaluated.

2.2.6 Metal analysis

A GBC 909 atomic absorption spectrometer (AAS) was used for all metal analysis. The application of AAS for metal analysis from biological specimens is reviewed by White and Gadd (1995) and O'Halloran *et al* (1997).

2.2.7 Scanning electron microscopy of polyacrylamide immobilized yeast

Specimens were incubated in cold 2.5 % glutaraldehyde in 0.1 M phosphate buffer for 12 h. This was followed by two washes in phosphate buffer and then a 30% to 100 % ethanol dehydration sequence, each of 10 min duration. The specimens were placed in 1,1,1,3,3,3-hexamethyl disilazane. The 1,1,1,3,3,3-hexamethyl disilazane wash was repeated three times for critical point drying. The specimens were mounted on metal stubs, gold coated in a sputter chamber and observed using a JEOL JSM-80 scanning electron microscope.

2.3 RESULTS

2.3.1 Effect of polyacrylamide gel on metal removal

Metal uptake by the polyacrylamide gel is presented in Appendix 1. Although mostly negligible, the binding of metals to the gel was accounted for in the analysis of biosorption data.

2.3.2 Metal removal over time

The immobilized *S. cerevisiae* efficiently removed all the metals investigated from solution. Copper uptake progressively increased up to 20 minutes, reaching saturation at 65 % metal removal (Refer to Figure 2.1). The initial concentration was 200 μmol/ℓ and the bioremediation process reduced the copper well within required drinking water criteria. The biosorbent's ability to remove the other metals was compared to its performance relative to copper and thus an initial concentration of 200 μmol/ℓ was used throughout these investigations.

Cobalt removal reached an equilibrium of 65-70% in 5 minutes (Refer to Figure 2.2). A zinc removal equilibrium was reached in 10 minutes of incubation and the percentage removal was 60 %. Cadmium removal was high at ≥ 88 % however equilibrium was relatively slow at 30 minutes. Gold removal was both rapid and high, with a removal of 75 % within the first 3 minutes and reaching 90% within 5 minutes of incubation.

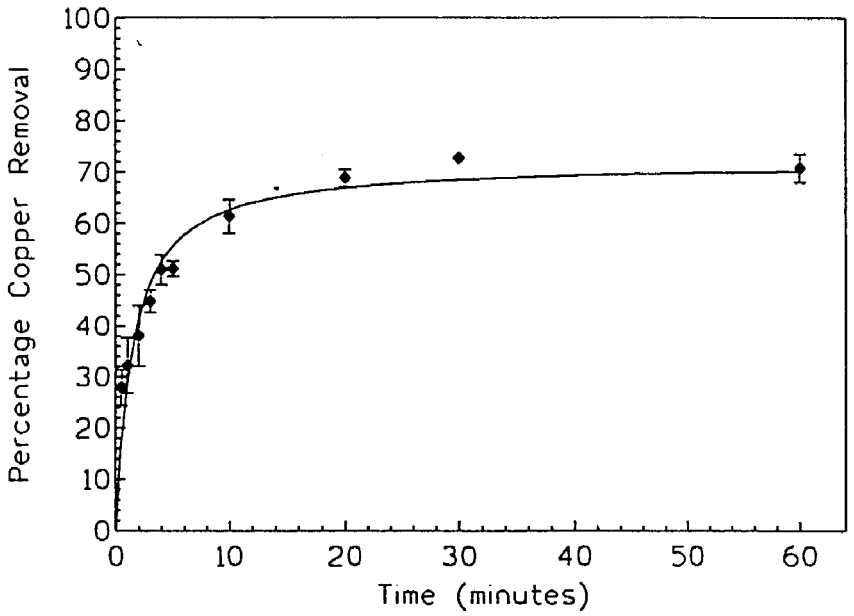

Figure 2.1: Percentage copper accumulated in batch reactors by immobilized *Saccharomyces cerevisiae* as a function of time (n=5, ± SD). Initial copper concentration was 200 μmol/ℓ.

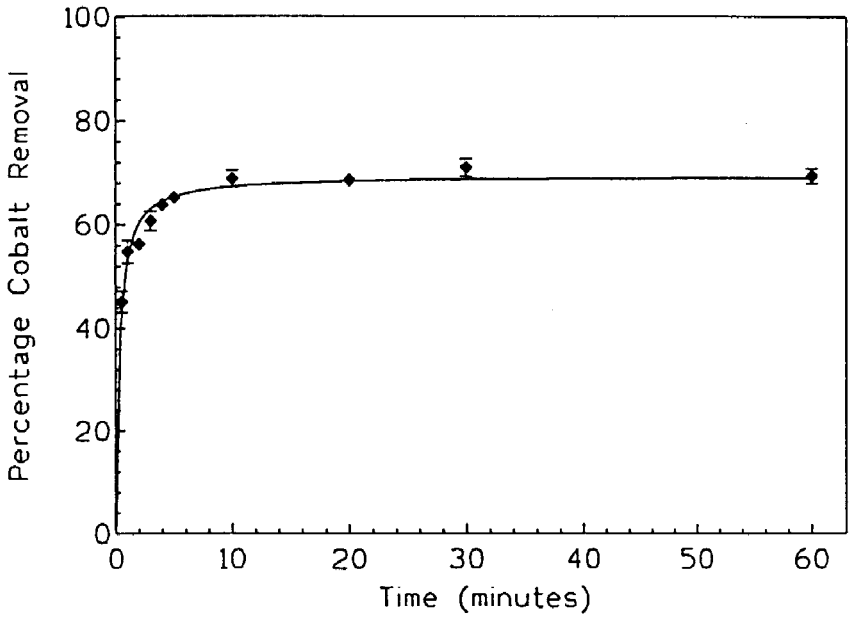

Figure 2.2: Percentage cobalt removed in batch reactors by immobilized *Saccharomyces cerevisiae* as a function of time (n=5, ±SD). The initial cobalt concentration was 200 μmol/ℓ.

Chromium and nickel removal was similar to copper removal, with equilibriums of 65 to 73 % being reached within 20 minutes of contact. Results from these experiments are not shown as the profiles are similar to those shown for copper and cobalt and only differ in the relative times and amounts of uptake.

2.3.3 Effect of pH on bioaccumulation

The bioaccumulation of divalent cations is pH dependant. By adjusting the pH of the metal solutions prior to biosorbent contact, a profile of optimum pH for metal removal was generated. Copper (Refer to Figure 2.3), chromium and nickel (results not shown) removal from solution by *S. cerevisiae* was maximized in the pH range 3 to 5 (60-70%), and was substantially reduced at pH 2 to≤ 7%. Zinc removal was not effected between pH 3 and 6, but only 34 % was accumulated at pH 2 as shown in Figure 2.4. Cobalt (Refer to Figure 2.5) and cadmium removal was high (≥ 70%) between pH 4 and 6, but was decreased below pH 4. At pH 2 removal was 5 and 25 % respectively for cobalt and cadmium. Gold was effectively removed from solution at lower solution pH values. At pH 3 and below removal was in excess of 80 % (Refer to Figure 2.6).

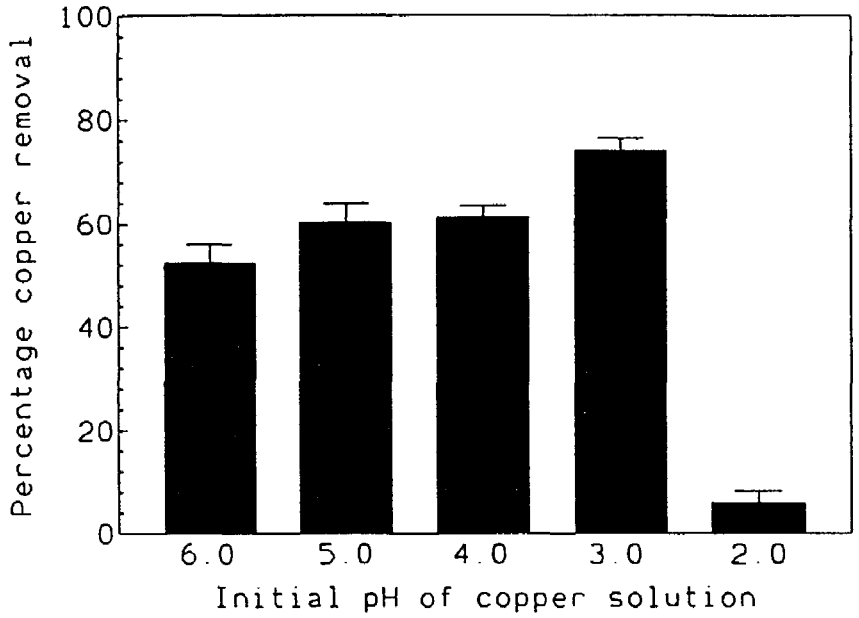

Figure 2.3: Removal of copper from aqueous solutions of varied pH in batch reactors by immobilized *Saccharomyces cerevisiae* (n=5, ± SD). The pH of the copper solutions was adjusted using HCl and NaOH and the initial concentration was 200 μmol/ℓ.

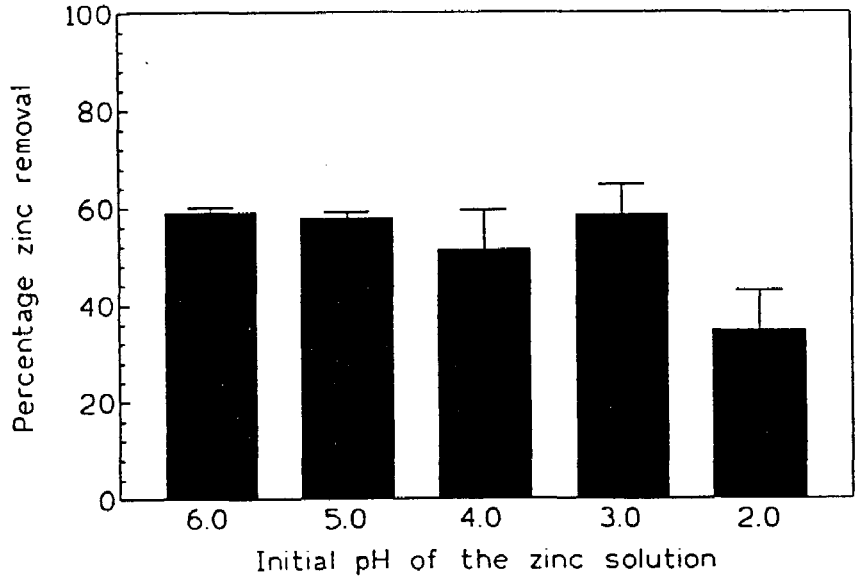

Figure 2.4: Removal of zinc from aqueous solutions of varied pH by immobilized *Saccharomyces cerevisiae* (n=5, ±SD). The pH of the zinc solutions was adjusted using HCl and NaOH and the initial concentration was 200 μmol/ℓ.

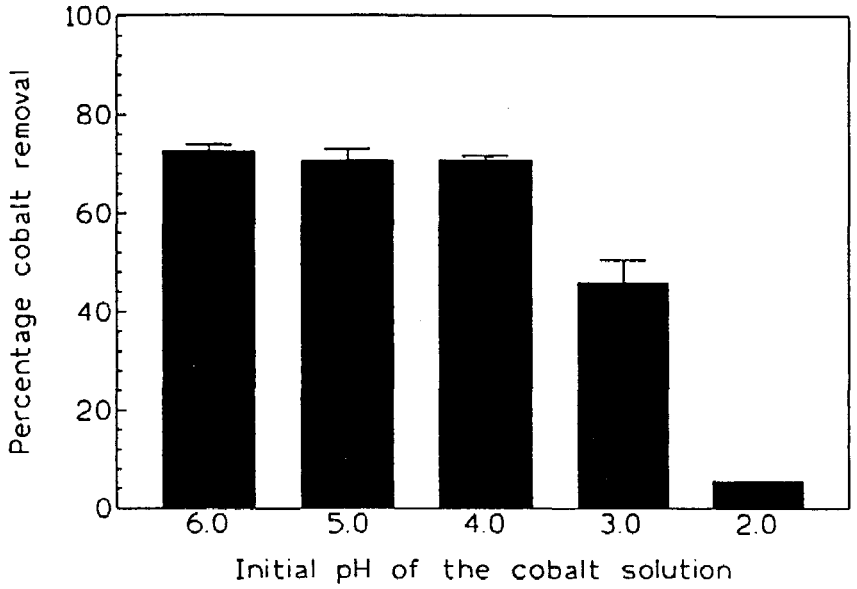

Figure 2.5: Removal of cobalt from aqueous solutions over a pH range in batch reactors by immobilized *Saccharomyces cerevisiae* (n=5, ± SD). The pH of the cobalt solutions was adjusted using HCl and NaOH and the initial concentration was 200 μmol/ℓ.

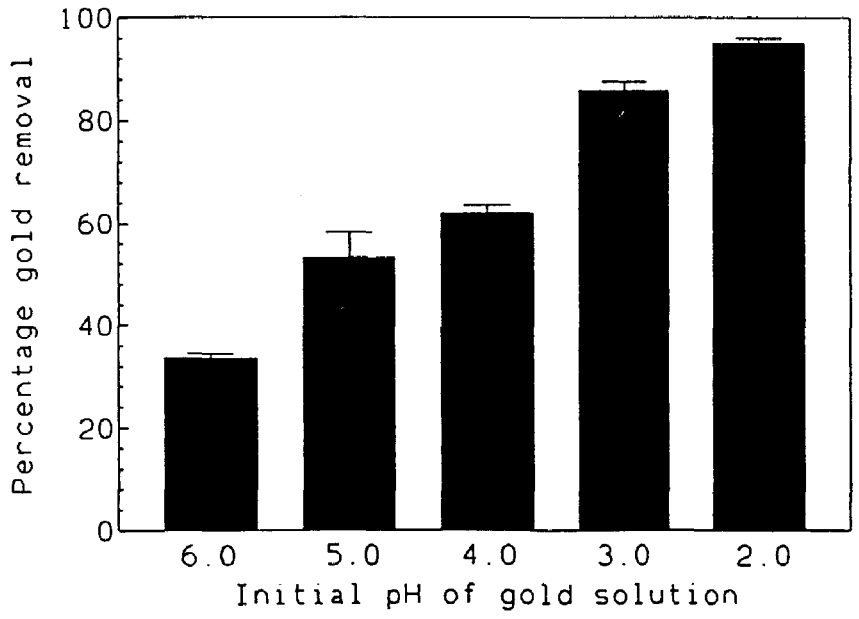

Figure 2.6: Removal of gold from aqueous solutions over a pH range in batch reactors by immobilized *Saccharomyces cerevisiae* (n=5, ± SD). The pH of the gold solutions was adjusted with HCl and NaOH and the initial concentration was 200 μmol/ℓ.

2.3.4 Sorption isotherms

Equilibrium binding isotherms were generated over an initial metal concentration range of 100 to 2000 μmol/ℓ. The metal solutions were initially pH 5 with the exception of gold which was at a pH 2. Biosorption followed Michaelis-Menten kinetics with the exception of zinc. Adsorption capacities and dissociation constants of metal equilibrium were calculated by linear transformation of binding data using the Hanes-Woolf plot and confirmed using Scatchard plots. Kd and B_{max} are given in Table 2.1. Equilibrium biosorption isotherms of copper, cadmium and gold are presented in Figure 2.7, Figure 2.8 and Figure 2.9, respectively.

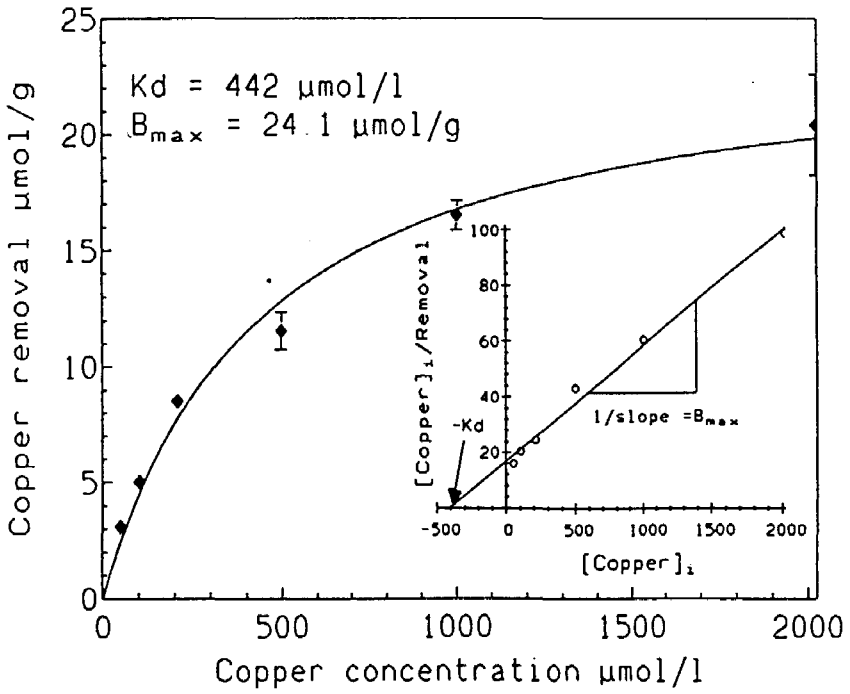

Figure 2.7: A sorption isotherm of copper removal by immobilized *Saccharomyces cerevisiae* in batch reactors (n=5, ± SD). The inset is a Hanes-Woolf plot, Kd and B_{max} were calculated as 442 μmol/ℓ and 24.1 μmol/g respectively.

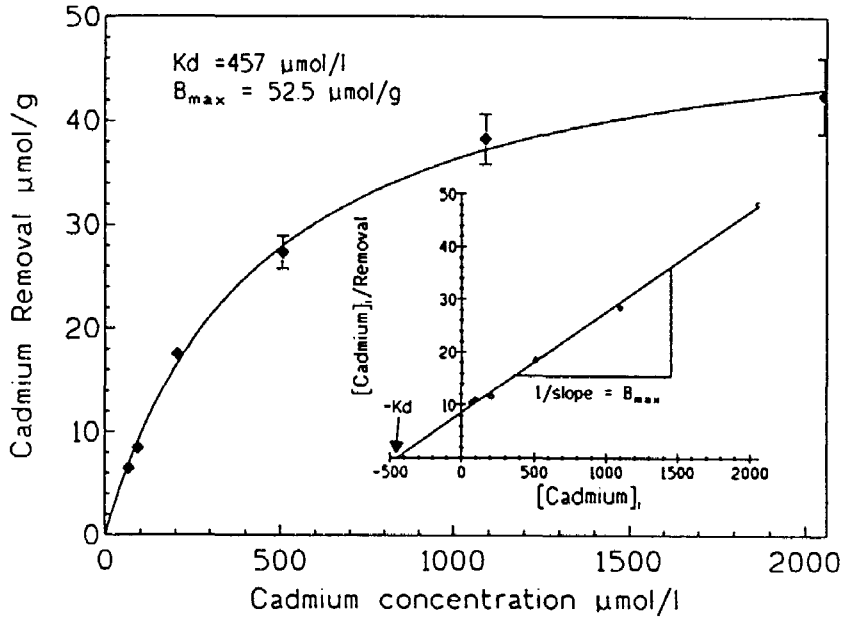

Figure 2.8 : A sorption isotherm of cadmium removal from aqueous solutions by immobilized *Saccharomyces cerevisiae* in batch reactors (n=5, ± SD). The inset is a Hanes-Woolf plot, Kd and B_{max} were calculated as 457 μmol/ℓ and 52.5 μmol/g respectively.

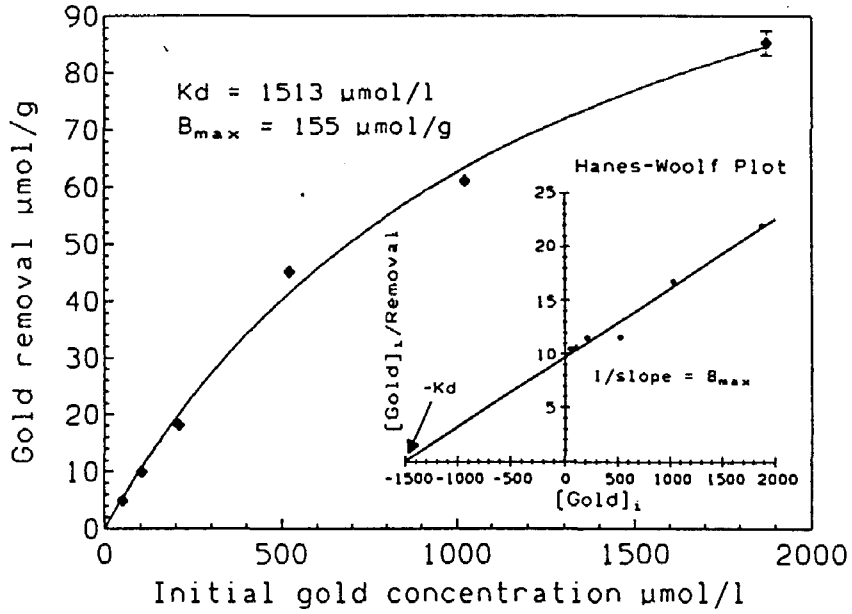

Figure 2.9: A sorption isotherm of gold removal from aqueous solutions by immobilized *Saccharomyces cerevisiae* in batch reactors (n=5, ± SD). The inset is a Hanes-Woolf plot, Kd and B_{max} were calculated as 1513 μmol/ℓ and 155 μmol/g respectively.

Table 2.1: Biosorption capacities of metals by *S. cerevisiae* as generated from Hanes-Woolf transformation plots.

METAL	Kd (μmol/ℓ)	B_{max} (μmol/g)
Copper	413	24.1
Nickel	151	19.5
Cadmium	457	52.5
Cobalt	500	49.6
Gold	1513	155
Chromium	144	20.2

2.3.5 Scanning electron microscopy

Scanning electron micrographs of meshed polyacrylamide, immobilized *S. cerevisiae* and immobilized *S. cerevisiae* exposed to gold prior to fixation are shown in Figure 2.10, 2.11 and 2.12, respectively. The polyacylamide micrograph reveals the open structure of the gel and a large surface area for cell entrapment. The immobilized control cells have a diameter of 4 to 5 μm and have a coiled twisted appearance. The cells incubated in a gold solution have similar features to the control cells. The dehydrated folded appearance of the immobilized cells is possibly a product of the immobilization process. The large indentations in the polyacrylmide gel containing the immobilized cells is possibly due to cell respiration or from cells which have been sheared off during the preparation stage. Scanning electron micrographs of cells from a free suspension have a smoother cell wall appearance (Refer to Figure 1.2).

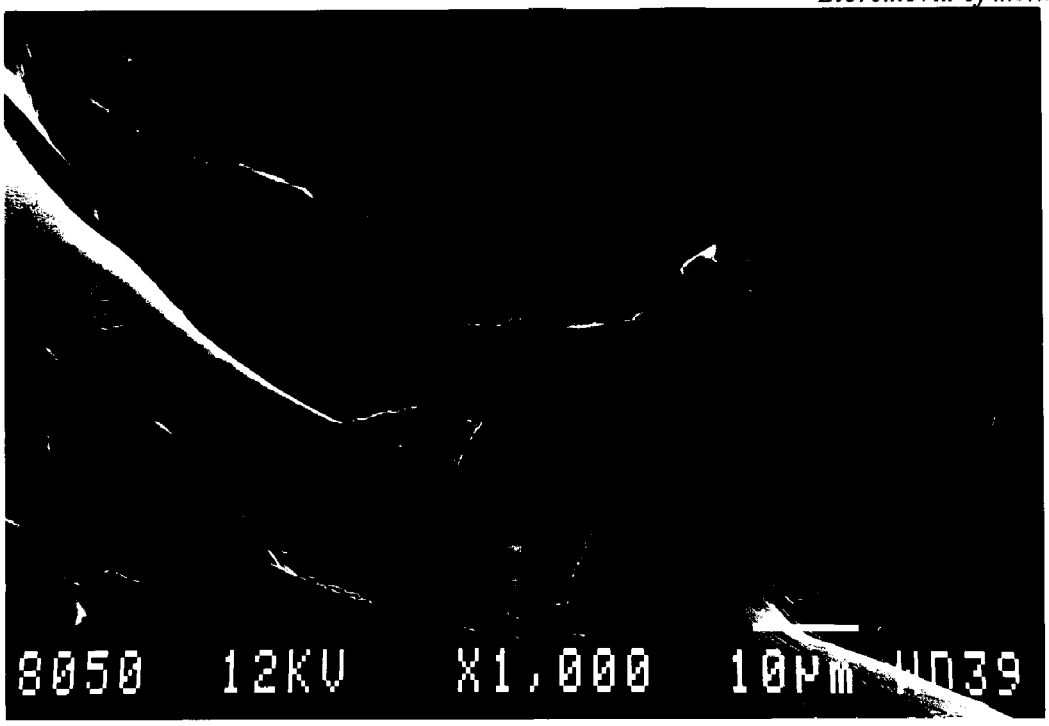

Figure 2.10: A scanning electron micrograph showing the open porous structure of a section of meshed polyacrylamide gel.

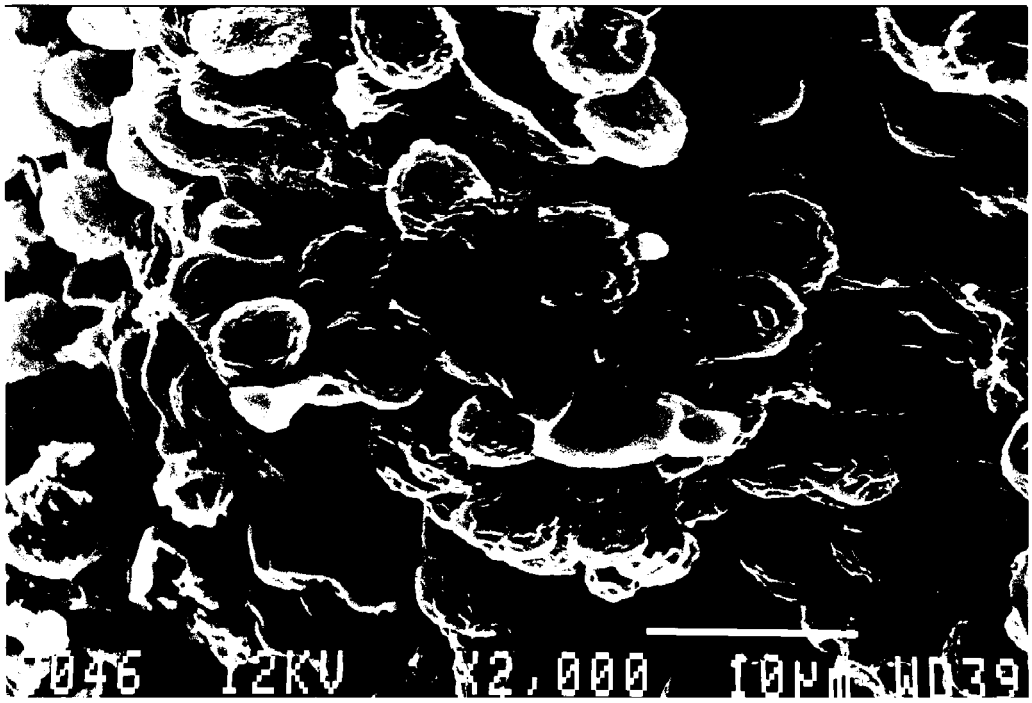

Figure 2.11: A scanning electron micrograph showing control *Saccharomyces cerevisiae* cells immobilized in a polyacrylamide gel matrix.

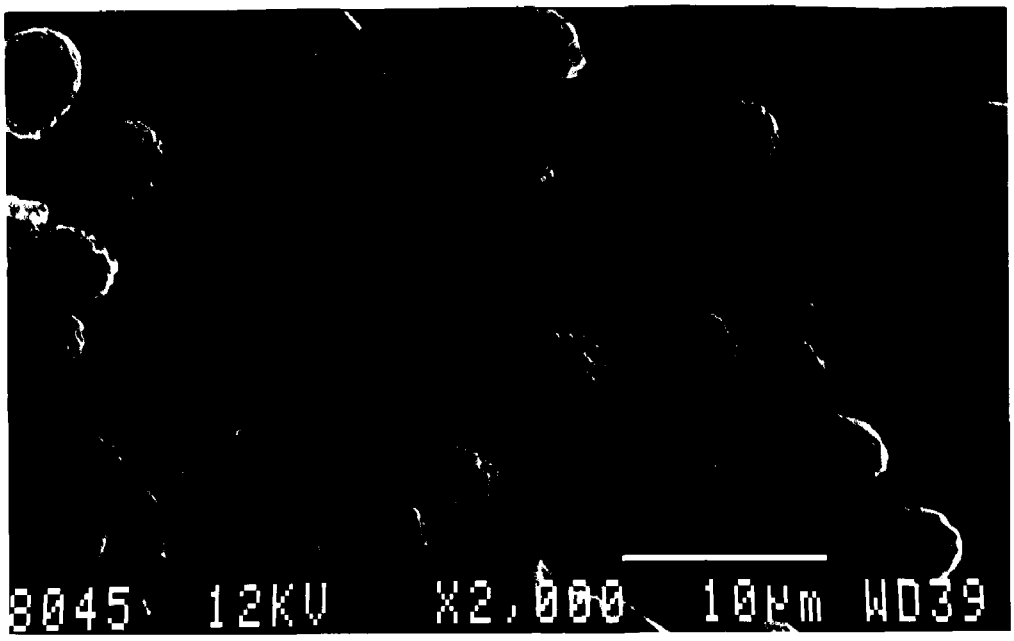

Figure 2.12: A scanning electron micrograph of *Saccharomyces cerevisiae* cells incubated in a gold solution prior to immobilization in a polyacrylamide gel matrix.

2.4 DISCUSSION

Metal uptake was rapid with gold and cobalt uptake reaching saturation within 5 minutes of contact. The binding of copper, zinc and nickel progressively increased up to 20 minutes reaching saturation at an uptake of 60 to 70 %. Cadmium removal, although high (88 %), was slower reaching an equilibrium in 30 minutes. This is similar to the findings of other researchers. Volesky *et al* (1992) reported high cadmium removal by free suspensions of baker's yeast with 75 % of sorption completed in less than 5 minutes. Rapid binding is most likely associated with metabolism-independent adsorption to the cell wall. Strandberg *et al* (1981) found uranium accumulated extracellularly on the surfaces of *S. cerevisiae* with the rate and extent of accumulation being dependent on environmental parameters. Uptake of the metals copper, cadmium and zinc by immobilized *Aphanocapsa pulchra* was above 75 % with a retention time of 5 minutes (Subramanian *et al*, 1994). Wehrheim and Wettern (1994) reported that more than 90 % of metal with an initial concentration range of 5 to 100 μmol/ℓ accumulated by *Chlorella fusca* was surface bound within the first minute of contact. Using a copper sensitive

probe, De Rome and Gadd (1987) found copper uptake by *S. cerevisiae* was initially rapidly bound to cell surfaces followed by intracellular uptake. Gadd and Mowell (1983) observed a total loss of viability at cadmium concentrations above 50 μmol/ℓ and suggested a metabolism-independent uptake mechanism by *Saccharomyces cerevisiae* at toxic metal concentrations.

Removal of zinc, chromium, cadmium, cobalt and nickel was enhanced at pH 4 and 5 and substantially reduced at pH 2. Copper removal remained high at pH 3 but was also reduced at pH 2. These results are consistent with those described by several authors (Ramelow *et al,* 1996; Sağ *et al,* 1995; Hoa *et al,* 1993; Vymazal, 1987). Ramelow *et al* (1992) found the bioremediation of copper, zinc, lead, cadmium and nickel was strongly pH dependent with a minimum binding at pH 2, but increased rapidly with increased pH and reached a maximum at pH 5-6. Fourest and Roux (1992) achieved optimal binding of nickel and zinc by *Rhizopus arrhizus* at neutral pH. The decreased binding of metal cations at lower pH indicates a possible recovery protocol for bound metals.

Binding of gold was highest at lower pH values. Gold and silver have been reported as only slightly effected by pH and in an opposite manner to the other metals, and maximum chromium (IV) adsorption rates by *S. cerevisiae* were at pH 1.0-2.0 at 100 mg/mℓ concentrations, indicating the importance of the metal speciation (Nourbakhsh *et al;* 1994). *Sargassum natans* was found to have a high capacity for gold accumulation which was optimal at pH $\leq$ 3 (Kuyucak and Volesky, 1988). The potential for gold removal at pH 2.5 from solutions containing uranium, lead and zinc was demonstrated by Kuyucak and Volesky (1988). The reverse pH optimum of gold removal relative to the other cations investigated may serve as an effective and selective removal strategy for gold solutions contaminated with competing metal cations. The ability to develop selective removal protocols for specific target metals will substantially enhance the viability of the bioremediation process. Manipulation of solution pH may provide a useful mechanism for selective metal recovery (Nui *et al,* 1993).

Guibal *et al* (1992) proposed the that the major influence of pH is due to its influence on the cell wall chemistry and metal solution chemistry. At pH ≈ 2, protonation of the cell wall restricts metal sorption. At pH 3-4 metal hydroxylation occurs which may limit diffusion and decrease uptake. The decrease in metal removal is most likely a result of H^+ ion competition for binding sites Fourest and Roux (1992) proposed a competitive mechanism for chelation between metals and protons at cell wall adsorption site at low pH. Similar observations were made by Huang *et al* (1988).

The binding isotherms followed Michaelis-Menten kinetics with the possible exception of zinc. Hanes-Woolf and Scatchard plots of the zinc binding data suggested biphasic uptake. Maximum uptake capacity of gold was the highest at 155 μmol/g. Cadmium and cobalt removal was relatively high at 52.5 μmol/g and 49.6 μmol/g respectively. Nickel, chromium and copper removal was relatively low at 19.5 μmol/g, 20.2 μmol/g and 24.1 μmol/g respectively. The maximum binding capacity (B_{max}) is useful for determining the biosorbent's potential removal capabilities at high metal concentrations. The dissociation constant corresponds to the half-saturation of the biosorbent with a low Kd value indicating high affinity (Fourest and Roux, 1992). This affinity related constant may be a useful consideration in bioreactor design. For example, a biosorbent with a low Kd for a particular metal may be applicable to a reactor which has low contact times such as a biosorption column. These constants may only be of value when comparing different metals or biosorbents if all other parameters remain consistent.

Metal accumulated by immobilized *S. cerevisiae* increased with increasing metal concentrations until cell saturation. The results suggest metal removal was by accumulation to the cell wall, although intracellular uptake cannot be discounted. Should intracellular uptake be involved, the rate of uptake would be more rapid than suggested in the literature. This would be possible if the immobilization process or the exposure to the metals increased cell membrane permeability. Studies by Garnham *et al* (1992b) of cobalt and zinc removal by *Chlorella salina* found uptake to be biphasic and that metal accumulation in the second phase followed Michaelis-Menten kinetics. Cellular analysis

showed that the metal was bound to intracellular components and the cell wall. Higher concentrations of metal were detected in the vacuole than in the cytosol, accounting for the multi-phasic uptake. In the present investigation only one uptake phase was evident, with the exception of zinc. Should the predominant metal removal mechanism be adsorption to the cell wall, rapid desorption and high recovery and concentration of the accumulated metal would be possible.

The maximum uptake capacities of the biosorbent in this study are relatively low. The highest removal rate being for gold of 155 μmol/g. Kuyucak and Volesky (1988) reported removal rates of gold up to 10 fold higher using a seaweed biomass. The data in this investigation is reported relative to the wet weight due to the nature of the biosorbent. Results in the literature are mostly expressed as a dry weight as with the results reported by Kuyucak and Volesky (1988). The metal concentration ranges investigated, although considerably above accepted drinking water criteria, were also relatively low which is reflected in the quantitative metal removal capacities observed. The design of the study was to facilitate rapid removal and metal recovery, and biosorbent regeneration and reuse which is facilitated by surface binding. The main criterion by which the biosorbent was assessed was its ability to remove metals from solution to concentrations of acceptable levels. The immobilized *S. cerevisiae* was indeed found to remove metals from the aqueous solutions and provide water of potable quality.

Controls using polyacrylamide gel with no *S. cerevisiae* removed varying amounts of metal from solution. No zinc was accumulated from solution but there was a relatively high removal of copper: approximately 15 % (complete polyacrylamide controls are reported in appendix 1). These control values were accounted for in the data analysis. The binding of metal to the immobilizing gel matrix, although mostly negligible, increases metal removal and is of further advantage to cell immobilization.

Scanning electron micrographs of immobilized cells showed a coiled twisted outer cell surface. This appearance was consistent in the control cells and cells exposed to metal

solutions. Jirků (1995) observed a conversion of ellipsoid shaped free *S. cerevisiae* cells into rod shaped forms of immobilized cells. Brady *et al* (1994f) found immobilized cells of *S. cerevisiae* became convoluted and smaller after exposure to copper. The size of the cells and rounded shape of the cells were maintained in this study. The change in the outer cell surface compared to free cells was possibly due to the stress placed on the cells during the immobilization process. The polyacrylamide appeared porous in the micrographs with the cells contributing to the porosity by creating small cavities in the gel matrix.

2.5 CONCLUSION

Immobilized *S. cerevisiae* efficiently removed metals from aqueous solutions. The removal was relatively rapid with adsorption equilibriums being reached for all metals within 30 minutes of incubation. The process was pH dependent with the removal of the divalent cations investigated being most effective at solution pH's ≥ 3. Gold removal by the biosorbent was both high and rapid at pH ≤ 3. The isotherms followed Michaelis-Menten binding kinetics with the exception of zinc. The metal removal capabilities of the immobilized *S. cerevisiae* suggest the biosorbent is potentially suitable for use with relatively low metal concentration high volume effluents. Scanning electron micrographs showed that the cell surface was altered by the immobilization process but was unaltered by metal binding.

CHAPTER III

RECOVERY OF METAL ACCUMULATED BY IMMOBILIZED *Saccharomyces cerevisiae*

3.1 INTRODUCTION

The recovery of bioaccumulated metals has both environmental and economic implications. Biologically bound metals, although removed from the aqueous solution, remain a potential threat to the environment. Unlike organic contaminants which can be metabolised to relatively innocuous compounds, bound metals are concentrated in localised areas and are potentially more toxic in this form if not recovered (Summers, 1992). Metabolism-independent biosorption is mostly reversible and metals can be recovered by non-destructible protocols. Intracellular accumulation may necessitate destructive recovery such as incineration or dissolution in acids. Destructive recovery of metals is feasible if the biomass is freely available as a cheap waste product or if the metal has sufficient value (Gadd, 1990).

Recovered metals are of economic value and can be recycled and returned to industrial processes, potentially subsidising their remediation. The successful recovery of bound metals from different microorganisms by non-destructive methods using a range of eluting agents has been reported by several researchers (Galun *et al*, 1983; Kuyucak and Volesky, 1989; Kalid *et al*, 1993).

The technical application of a bioremediation operation would be substantially enhanced if the biomass could be regenerated and used for repeated adsorption-desorption applications (Tsezos, 1984). The ability to reuse the biomass would improve the economic benefits of a bioremediation industrial scale-up (McLean *et al*, 1994). Immobilized cells, which are mechanically more stable and maintain good adsorbing characteristics, have better regeneration properties than free cells in suspension. Nakajima

and Sakaguchi (1993) reported that immobilized basidiomycetous cells were capable of 5 cycles of repeated uranium adsorption and desorption. This system was suitable for batch and column strategies. Gardea-Torresday *et al* (1996) subjected a biomass to 10 binding and elution cycles without significant loss to binding capacity.

The eluting solution should allow complete and rapid removal of metals bound to the biosorbent. It should be non-toxic and cause the least amount of damage to adsorption properties of the biomass and so allow reuse (Tsezos, 1984). The volume of eluting solution required should be small and should yield high metal concentrates.

This study investigated the ability of potential metal eluting agents to recover copper bound to immobilized *S. cerevisiae* biomass in batch reactors. A rapid technique for determining the potential reusability of the biosorbent was developed. The uptake capacity of the biosorbent and bound metal recoveries over repeated cycles were monitored to determine the reusability of the biosorbent. Electron microscopy studies were used to observe any morphological changes or damage to the cell during repeated use.

3.2 MATERIALS and METHODS

3.2.1 Materials

Commercial preparations of *S. cerevisiae* were obtained from Anchor Yeast Inc (production strain, 90 % cell viability). $CuCl_2.2H_2O$, $CaCl_2.2H_2O$, Ca_2CO_2 and $CaHCO_3$ were purchased from Merck. KOH, NaOH, KCl, H_2SO_4, HNO_3 and HCl were supplied by Saarchem. Ultra-pure deionised water was used in all experiments and was purified by a Milli-Q water system.

3.2.2 *S. cerevisiae* immobilization

Yeast was immobilized as described previously in 2.2.2.

3.2.3 Metal adsorption-desorption profiles

Immobilized biosorbent (1g) was weighed into conical flasks. A 10 mℓ copper chloride solution of 200μmol/ℓ was added. The reaction mixture was incubated over 30 minutes with shaking at ambient temperature and then centrifuged for 5 minutes at 500 g. A 1 mℓ sample of the supernatant was removed for determination of metal accumulation. The post contact reaction mixtures were treated with potential eluting solutions. The acids HNO_3, HCl and H_2SO_4 (0.01-0.1 M) sequentially decreased the pH and desorption profiles were generated. The volumes and concentrations of acid used were recorded. Ca_2CO_3, $CaHCO_3$, $CaCl_2.2H_2O$, KCl, KOH and NaOH solutions (1 M) were added to the reaction mixtures in aliquots of 0.1 mℓ, 0.5 mℓ and 1.0 mℓ.

Relevant control samples were run concurrently as decribed in 2.2.5. A 1 mℓ sample was removed for the determination of bound metal and the dilution effect of adding the eluting solution was accounted for in the calculation of recovery efficiency.

3.2.4 Repeated use of the biosorbent

Eight adsorption-desorption cycles were investigated in batch reactors. Refer to Figure 3.1 for the batch reactor reusability protocol.

3.2.5 Metal Analysis

Copper analysis was as described in section 2.2.6.

3.2.6 Transmission electron microscopy

Yeast cells were investigated by transmission electron microscopy to observe potential morphological changes of cells and to gain insight into binding mechanisms during adsorption-desorption of metals. Free cell suspensions of baker's yeast conditioned with HCl and NaOH washing as described in section 2.2.2 served as controls. Cells exposed to 200 μmol/ℓ copper in batch reactors for 30 min and cells exposed to copper and acid desorption cycles were prepared for transmission electron microscopy.

Fixation of *S. cerevisiae* was achieved by incubating the cells in 2.5 % gluteraldehyde in a 0.1 phosphate buffer solution for 12 h followed by 2 phosphate buffer washes and ethanol dehydration as described in section 2.2.7. A propylene oxide transition and a sequential resin embedding step were performed as described by Cross (1989). Trapezium shaped sections of 120 nm were cut using a RMC MT7 ultramicrotome. The cut sections were stained with 5 % uranyl acetate and lead citrate and then examined under a JEOL JEM 100 CX transmission electron microscope.

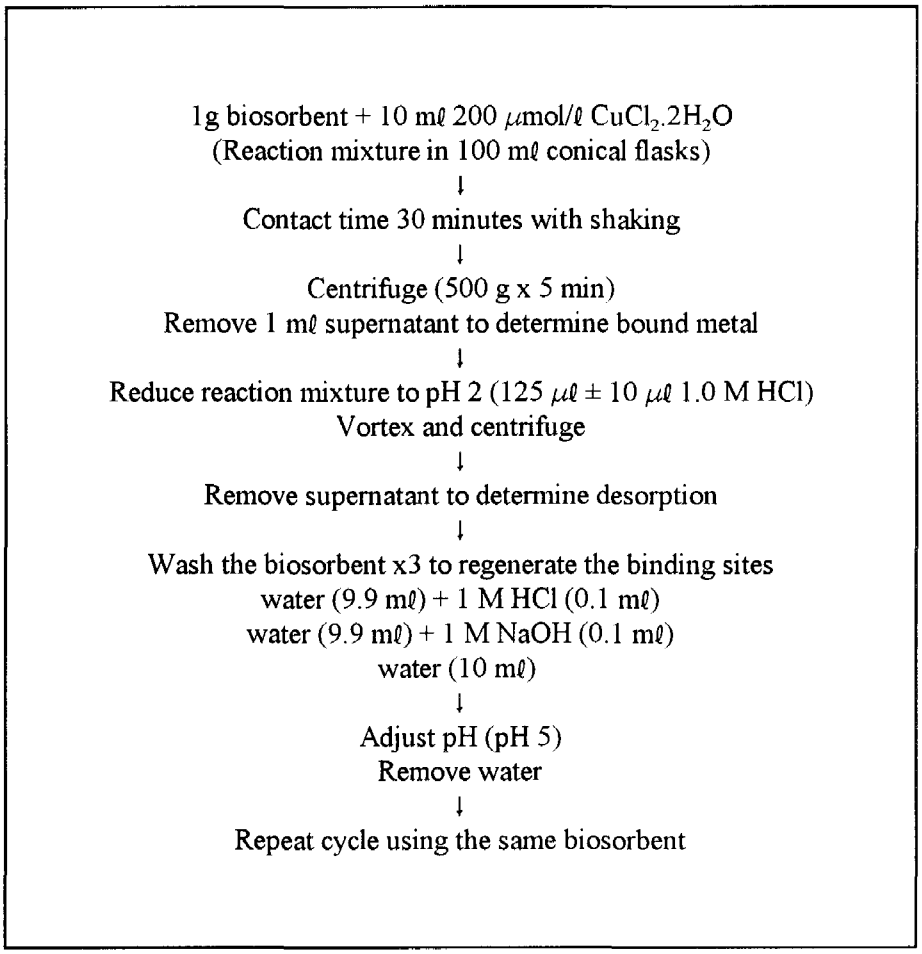

Figure 3.1 : A flow-diagram of the batch reactor adsorption-desorption protocol.

3.3 RESULTS

3.3.1 Copper recovery from the biosorbent

Copper recovery was most efficient using the mineral acids (Refer to table 3.1). By decreasing the pH a copper recovery of ≥ 80 % was achieved at pH 2. A typical pH desorption profile is shown in Figure 3.2, where bound copper was stripped from the biomass by sequentially decreasing the solution pH. HCl, H_2SO_4 and HNO_3 effectively desorbed bound copper by addition of small volumes of 1.0 M solutions (0.13, 0.2 and 0.25 mℓ respectively) to the remaining 9 mℓ reaction mixtures (Table 3.1). $CaHCO_3$, $CaCl_2.2H_2O$, Ca_2CO_3 and KCl were relatively ineffective at recovering copper and desorbed 32.1 %, 24.9 %, 14.3 % and 9.3 % respectively (Table 3.1). NaOH and KOH increased copper removal from solution.

Table 3.1 : Percentage desorption of copper from immobilized *S. cerevisiae* in batch reactors.

Desorption agent (1 μmol/ℓ)	Volume (mℓ)	Final concentration of desorption agents (μmol/ℓ)	Desorption %
H_2SO_4	0.20	0.021	85.0 ± 4.3[a]
HCl	0.13	0.014	80.4 ± 5.5[a]
HNO_3	0.25	0.027	89.5 ± 4.3[a]
$CaHCO_3$	1.0	0.1	32.1 ± 6.1[a]
Ca_2CO_3	1.0	0.1	14.3 ± 2.6[a]
KCl	1.0	0.1	9.3 ± 1.8[a]
$CaCl_2.H_2O$	1.0	0.1	24.9 ± 4.2[a]
Water control	0.5	***	1.2 ± 0.8[a]

a - Mean ± SD of 5 determinations

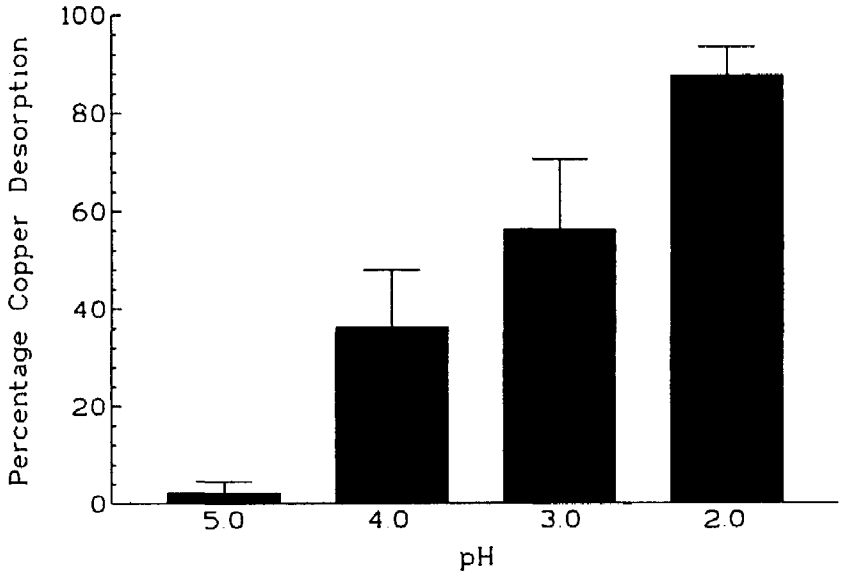

Figure 3.2: Desorption of copper accumulated to immobilized *Saccharomyces cerevisiae* (n=4, ± SD). The desorption profile was generated by sequential reduction of the pH using dilute HCl.

3.3.2 Adsorption-desorption cycles

Adsorption-desorption was investigated over 8 cycles to assess the reusability of the immobilized *S. cerevisiae*. Copper removal from solution in batch reactors increased from an initial 65 % to between 70-85 % efficiency in the latter cycles. (Figure 3.3). Desorption was achieved by reducing the pH using HCl. Recovery of metal was high with an average recovery of ≥ 85 % over the repeated cycles. No apparent damage was done to the biosorbent and no adverse effect on uptake or recovery of the metal was observed. The immobilized biomass was easily recovered and regenerated for reuse.

Adsorption-desorption studies using free cell suspensions were also attempted as a comparison but were unsuccessful due to the loss of cells between each cycle.

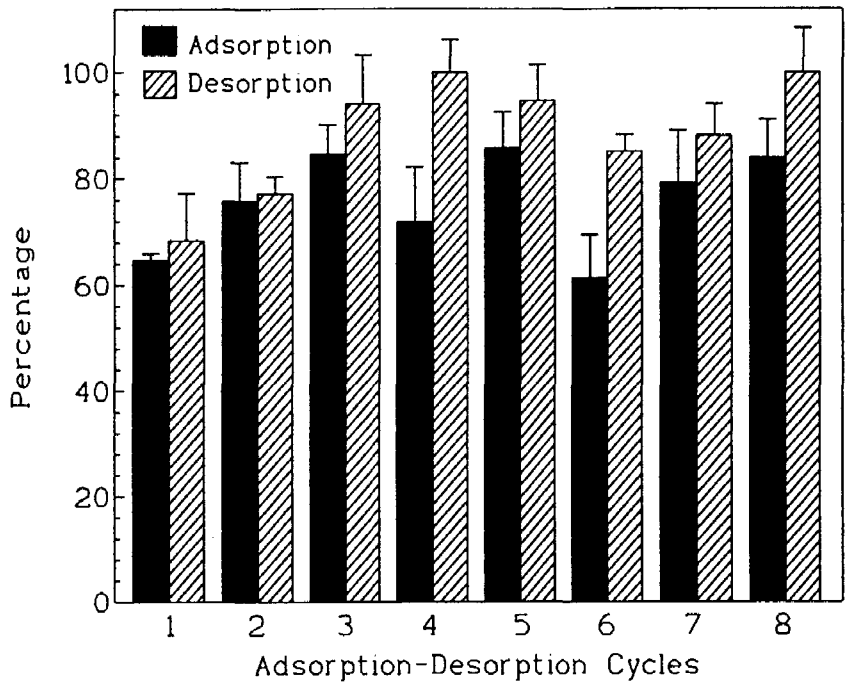

Figure 3.3 : Eight repeated adsorption-desorption cycles in batch reactors determined the reusability of the immobilised *Saccharomyces cerevisiae* biosorbent (n=5, ± SD). The initial copper concentration was 200 μmol/ℓ and desorption was acheived using HCl.

3.3.3 Transmission electron microscopy of *S. cerevisiae*

Cells exposed to copper and dilute acid treatment were observed under transmission electron microscopy to determine the extent of any structural damage to the cell integrity, morphological changes and to assess the type of bioaccumulation. Untreated cells were used as controls and compared to copper and acid treated specimens (Refer to figure 3.4). From the randomly selected micrographs no damage to the metal exposed cells was detected. Characteristic of the cells exposed to copper were dark electron dense walls (Refer to Figure 3.5). Cells exposed to adsorption-desorption cycles were more spherical in appearance than the cells only exposed to copper, and the cell walls were not as electron dense (Refer to Figure 3.6).

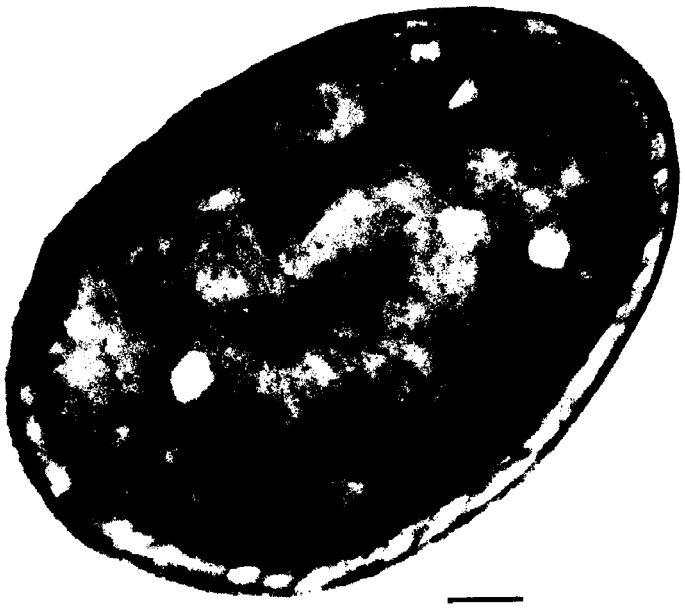

Figure 3.4: A transmission electron micrograph of a yeast cell. *Saccharomyces cerevisiae* cells were washed in d.d. milli-Q water and prepared as controls. Scale bar = 0.5μm.

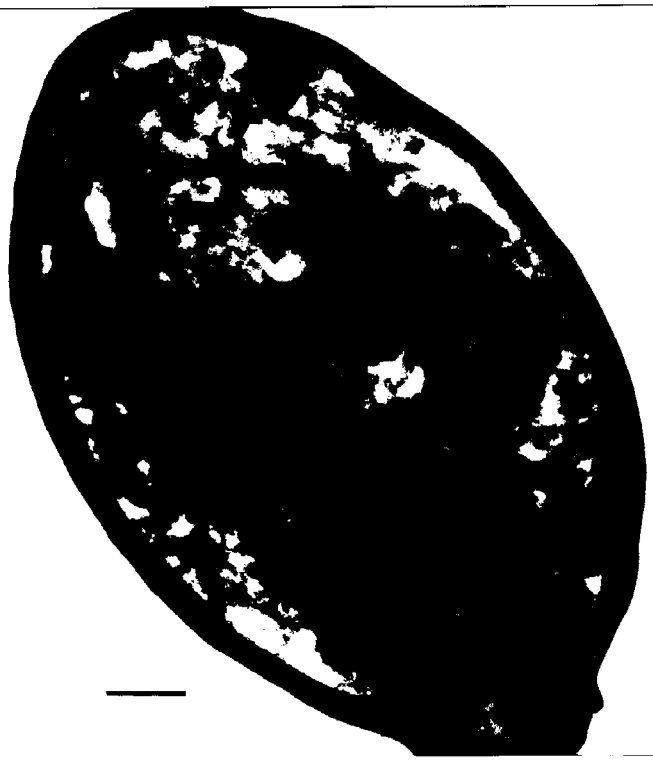

Figure 3.5: A transmission electron micrograph showing a cross-sectional view of a *Saccharomyces cerevisiae* cell. The cells were prepared for electron microscopy after a 30 minute exposure to copper chloride. Scale bar = 0.5 μm.

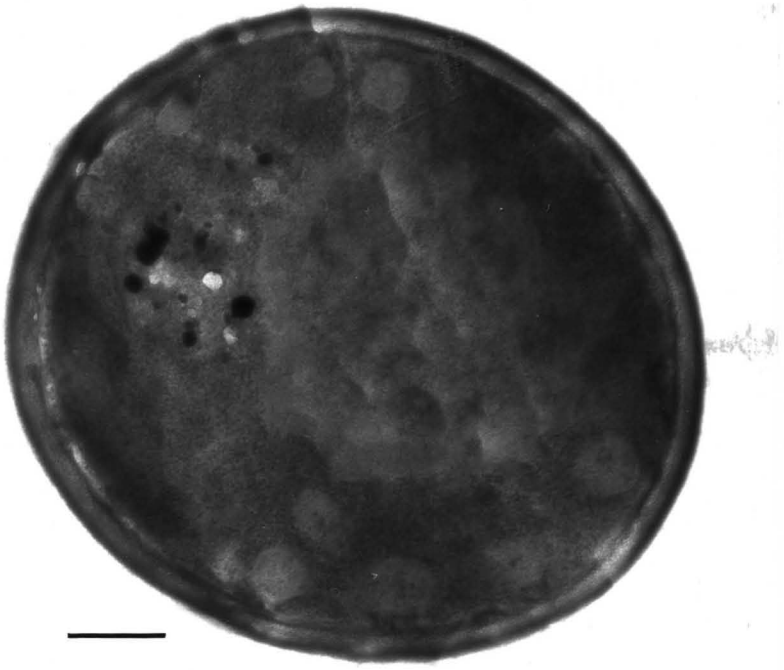

Figure 3.5: A transmission electron micrograph showing a cross-sectional view of a *Saccharomyces cerevisiae* cell. The yeast cells were prepared for electron microscopy after a 30 minute exposure to copper chloride followed by acid treatment to release bound metal. Scale bar = 0.5 μm.

3.4 DISCUSSION

Metal recovery was most efficient using the mineral acids. By sequentially decreasing the reaction mixture pH with nominal volumes of HCl, H_2SO_4 and HNO_3 a copper recovery of ≥ 80% was achieved. Similar results were obtained in the desorption of copper from *Streptomyces noursei* by mineral and organic acids (Mattuschka and Straube, 1993). Bux *et al* (1995) found HCl was most efficient for chromium, cadmium and zinc desorption from activated sludge, and acetic acid for copper and nickel. Shuttleworth and Unz (1993) recovered up to 75 % of bound nickel and zinc by placing the metal loaded cells in a $CaCl_2$ solution. They suggested an ion exchange process. Garnham *et al* (1992a) reported the desorption of cobalt from loaded, immobilized *Chlorella salina* biomass by decreasing the pH and increasing the concentration of cations. The authors also suggested that desorption was due to an exchange of bound cobalt for H^+ or the cations. Minimum quantities of dilute eluting solutions were used. The advantage of dilute acid elution is the

low chemical stress to the cells (McLean *et al*, 1994). The high metal recovery supports the proposed metal removal mechanism of adsorption to the cell wall of the yeast cells. The calcium carbonates, calcium chloride and potassium chloride were ineffective as eluting solutions. NaOH and KOH increased adsorption. NaOH was used for biosorbent regeneration post acid desorption. It neutralised the biosorbent pH and had the beneficial side-effect of enhancing metal removal efficiency.

Adsorption-desorption of copper was investigated over 8 cycles in batch studies with no apparent adverse effect on uptake capacity or recovery. The ability to recycle the yeast enhances its potential for continuous and scale-up bioremediation operation. The ability for repeated use of immobilized biosorbents was reported by Harris and Ramelow (1990). Copper was desorbed from *C. vulgaris* and *S. quadricauda* using 0.05 M sodium acetate at pH 2. Up to eight cycles of copper adsorption-desorption cycles were run. All the copper was recovered from *S. quadricauda* with two sodium acetate washes per cycle and recovery from *C. vulgaris* was 63 % . Purified cell walls of *Baccillus subtilis* exposed to repeated copper-HNO_3 adsorption-desorption increased copper uptake over 5 cycles due to enhanced exposure of binding sites (McLean *et al*, 1994). The major implication of a reusable biosorbent is that it substantially improves the economics of the bioremediation system. The reusable biosorbent has the potential to provide an effective, environmentally friendly, metal purification technology.

Investigations of the reusability of free cell suspensions of *S. cerevisiae* were inconclusive due to the high cell loss during the regeneration process. Similar observations were made by Nakajima and Sakaguchi (1993) who reported a 50 % decrease in dry cell weight during 5 adsorption-desorption cycles. Immobilized cells mass decreased by only 3 % during 5 cycles of a corresponding investigation.

Transmission electron microscopy was used to determine the effect of metal and acid exposure to the cell structure. Tsezos (1984) found mineral acids caused substantial damage to *R. arrhizus* biomass. In this study cells exposed to copper and acid treatment

showed no apparent damage to cell integrity, neither were any morphological changes observed when comparing these cells to untreated controls. The micrographs of cells exposed to copper were characterized by dark, electron dense cell walls, which may indicate copper binding.

A limitation of transmission electron microscopy as a method for observation of metal binding is the inability to positively identify the metals which appear on the image as dark electron dense areas. Wong and So (1993) observed that cells exposed to copper retained the metal on the cell surface, however, cells not exposed to copper did not show any image under electron microscope. Beveridge and Murray (1976) reported that metals with atomic numbers greater than 11 could be detected by electron microscopy on the cell walls of *Bacillus subtilis*. Their findings were supported by X-ray diffraction data. Golab (1991) presented electron micrographs of *Streptomyces sp* showing lead bound to the cell surface. The binding of metal to the cell wall would be expected as it comprises many potential negative sites for cationic deposition.

The micrographs of cells exposed to copper followed by acid treatment were spherical in appearance and the cell walls were not as electron dense as those cells only exposed to copper. The micrographs suggest a metabolism-independent surface binding which would allow rapid adsorption and recovery of bound metal and thus repeated use of the biosorbent would be possible.

3.5 CONCLUSION

Copper accumulation by immobilized *S. cerevisiae* biomass was reversible and was most successfully recovered using HCl, H_2SO_4 and HNO_3. A rapid method for assessment of biomass reusability showed the yeast biosorbent to be reusable. Removal and recovery efficiencies of the immobilized biosorbent improved with up to 8 repeated adsorption-desorption cycles in batch reactors. The metal uptake capacity was maintained with reuse of the biosorbent which would potentially lower costs if bioremediation were to be applied

to industrial effluent. Electron micrographs showed no damage to the cells and appeared to support the mechanism of metal binding to the cell surface. The biosorbent showed the potential for reuse in continuous bioremediation systems. Biosorption columns were investigated in the following chapter as a system for reusing the immobilized *S. cerevisiae* without the constraint of solid/liquid separation procedures.

CHAPTER IV

METAL REMOVAL AND RECOVERY FROM *Saccharomyces cerevisiae* BIOSORPTION COLUMNS

4.1 INTRODUCTION

Immobilized biomass in packed-bed columns have shown potential as bioreactors for metal removal and recovery. The main advantage of a fixed-bed reactor is ease of use due to convenient liquid/solid separation. Fixed-bed reactors offer an applications orientated approach which allows efficient utilization of the sorbent resulting in better quality eluents (Volesky and Prasetyo, 1994). The biosorbent should have a high affinity for the target metal for rapid uptake. The efficiency of immobilized bioreactors are dependent on the flow rate, bead size and the composition of the medium. The biosorption columns may require optimization to obtain maximum metal removal at the highest possible flux rates (Chein and Sofer, 1987). These biosorption columns may be highly efficient reactors for metal removal as demonstrated by Pümpel and Schinner (1993). Application of 1 mmol/ℓ solutions of silver, copper and lead to immobilized *Penicillium sp* biosorption columns resulted in eluants of $\leq 1\mu$mol/ℓ, representing a metal removal of $\geq$ 99 %.

On the application of metal, biosorption columns become saturated in the direction of the metal solution flow (Volesky, 1987). At the point of column saturation, the accumulated metals can be recovered by eluting the column with an appropriate desorbing solution. The recovery of metal and biomass regeneration have important financial implications for the feasibility of the bioremediation technology. Recovery is necessary for complete removal of the metal from the biosphere and for metal recycling. Metal elution from a biosorption column should produce a low volume, high concentration eluent to maximize metal recovery and minimize cost (Tsezos *et al*, 1989). The elution process can be optimized by determining the optimum eluting solution concentration and volume, and the

optimum flow rate. Tsezos *et al* (1989) found the elution process less efficient at higher flow rates which required larger volumes of the eluting solution.

The biomass can be regenerated after metal desorption which permits recycling (Norberg and Rydin, 1984). Biosorbents may be regenerated by elution with an appropriate washing solution to remove the residual desorbing solution. One or two bed volumes of water may be adequate for this process depending on the desorbing solution used. Sharma and Forster (1995), using 1.0 M NaOH to desorb chromium from peat, found biomass regeneration caused structural changes which affected the porosity and increased the resistance to flow. Their regeneration efficiencies were below 40 %. Ideal desorbing and regeneration protocols should not damage the biomass or cause swelling and should only require minimal eluent volumes. These protocols should also not create further environmental hazards.

Holan *et al* (1993) reported the adsorption-desorption of cadmium from immobilized *Ascophyllum nodosum* using 0.2 M HCl through 5 cycles with no changes in uptake capacity. Tsezos *et al* (1989) demonstrated the reusability of an immobilized *R. arrhizus* biosorbent in continuous packed-bed columns for up to 12 cycles with no apparent indication of failure.

The competition for binding sites and different binding affinities may lead to selective metal removal from mixed metal solutions by the biosorbent (Pümpel and Schinner, 1993). This may have potential for selective recovery of target metals from mixed metal solutions, a subject which has not being well explored for immobilized biosorbents. De Rome and Gadd (1991) reported on the recovery of uranium in the presence of caesium and strontium, with uranium almost selectively recovered. Selective recovery would have positive economic implications for bioremediation processes and it should be further investigated.

The objectives of this study were to investigate the binding of copper, cobalt, zinc,

cadmium, nickel, chromium (III) and gold (I and/or III) to immobilized *S. cerevisiae* in packed-bed semi-continuous flow columns, to determine the recovery and concentration potential and to assess the reusability of the biomass. Two columns in series were used to demonstrate the potential for selective binding and selective recovery from mixed metal solutions.

4.2 MATERIALS and METHODS

4.2.1 Materials

As given previously in 2.2.1.

4.2.2 *S. cerevisiae* immobilization

Yeast immobilization as described previously in 2.2.2.

4.2.3 Biosorption columns preparation

LKB chromatography columns of diameter 1.6 cm and 30 cm length were used. The adjustable columns were calibrated to 20 mℓ and packed with the immobilized yeast slurry. The biosorption columns were conditioned by passing 20 mℓ 0.1 M HCl, 10 mℓ 0.05 M NaOH and 20 mℓ water through the column. This was followed by single metal applications of 200 μmol/ℓ of metal and a volume of 500 mℓ was passed through the columns at a flow rate of 1 mℓ per minute. Metal chloride solutions investigated were; copper, zinc, cobalt, cadmium, nickel and chromium at pH 5. Gold was investigated at pH 2. The eluent was collected in 10 mℓ fractions.

The metals were then eluted from the columns with 40 mℓ 0.1 M HCl and collected in 2 mℓ fractions. The columns were reconditioned by washing with 20 mℓ 0.05 M NaOH and 20 mℓ water. The metal solutions were then reapplied and the process repeated. Chromium was eluted from the column using 40 mℓ 1 M HCl and the bioaccumulated gold was eluted using a 40 mℓ solution of 0.1 M $CaCl_2$ in 3 M HCl.

4.2.4 Selective recovery of metals from a mixed metal solution

A schematic presentation of the selective recovery process from two biosorption columns is presented in Figure 1. Mixed metal solutions of copper and cobalt; copper and zinc; and copper and cadmium were passed through two columns set in series. The columns

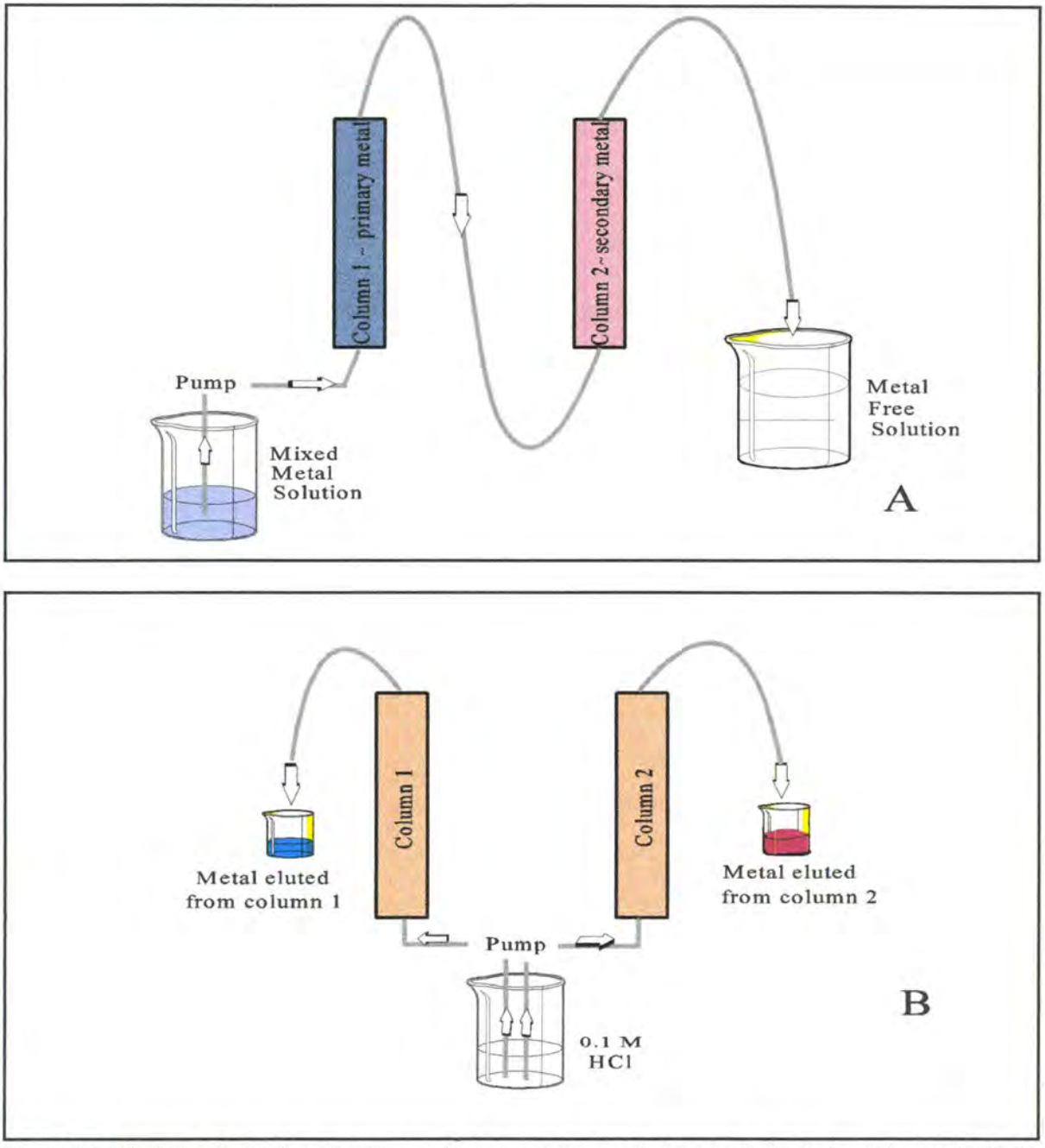

Figure 4.1: A) Two columns in series were used to separate two metals from a mixed solution. The metal with the higher affinity bound preferentially and saturated the first column; B) At saturation of the first column, the columns were disconnected and eluted with 0.1 M HCl, and the metals from the individual columns recovered.

were monitored for metal saturation. At saturation of the first column in the series, the columns were disconnected and the metals desorbed from the individual columns using 40 mℓ 0.1 M HCl.

4.2.5 Reusability of the biosorbent columns

Eight adsorption-desorption cycles were investigated for a biosorption column using a 200 μmol/ℓ copper solution. The metal application, desorption and column reconditioning were as described in section 4.2.3.

4.3 RESULTS

4.3.1 Metal uptake on immobilized *S. cerevisiae* biosorption columns

Examples of typical biosorption column bioaccumulation profiles are shown in Figures 4.2-4.5. The biosorbent removed the metals until reaching a saturation threshold, following which the uptake declined rapidly. The metals were desorbed and recovered with 0.1 M HCl elution. Removal of metals are reported as μmol/g of *S. cerevisiae* and recovery of bound metals as a percentage in Table 4.1. Above 90 % of the copper, zinc and cobalt recovered was concentrated in a 10 mℓ acid elution volume. A typical metal desorption profile is presented in Figure 4.6. Cadmium and nickel were recovered in approximately 15 mℓ acid eluent. The columns were reconditioned and the metals reapplied to assess reusability. Metal removal with the second application remained constant or in some cases was increased (Refer to Table 4.1). Chromium and gold were not desorbed using 0.1 M HCl. Increasing the acid concentration to 1.0 M yielded a 34 % chromium recovery. A gold recovery of 67 % from the biosorption columns was achieved using 0.1 M $CaCl_2$ in 3 M HCl. Gold and chromium were recovered in approximately 25 mℓ of eluent. Due to the low recovery of these two metals a second metal application was not performed.

Photographs of the desorption of a copper saturated biosorption column are presented in Figures 4.7 to 4.9.

Table 4.1 : Metal uptake and recovery on a *S. cerevisiae* column.

METAL	Removal* µmol/g**		Recovery* percentage	
	Run 1	Run 2	Run 1	Run 2
Copper	27.9	27	100	91
Cobalt	49.7	50	100	99
Cadmium	31.6	37.3	100	100
Nickel	33.7	38.7	100	91
Zinc	40.7	49.9	89	61
Chromium	28.6	---	34***	---
Gold	42.2	---	67****	---

* Typical results reported

** Bioaccumulation results expressed as µmoles/g wet weight *S. cerevisiae*

*** Chromium recovered with 1 M HCl.

**** Gold recovered with 0.1 M $CaCl_2$ in 3 M HCl

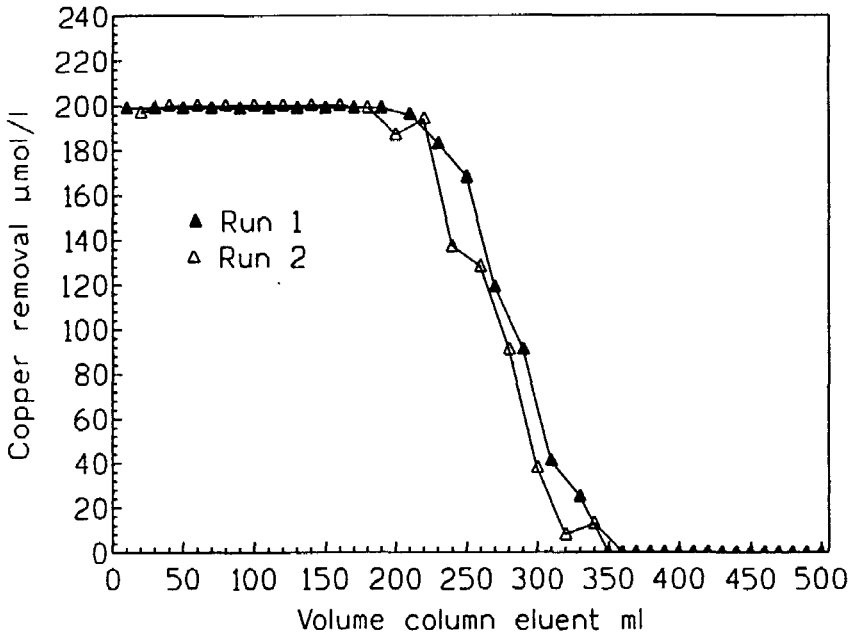

Figure 4.2: Bioaccumulation of copper on a 20 mℓ immobilized *Saccharomyces cerevisiae* column. The copper was desorbed using 0.1 M HCl, the column was reconditioned and the copper reapplied to determine reusability of the biosorbent.

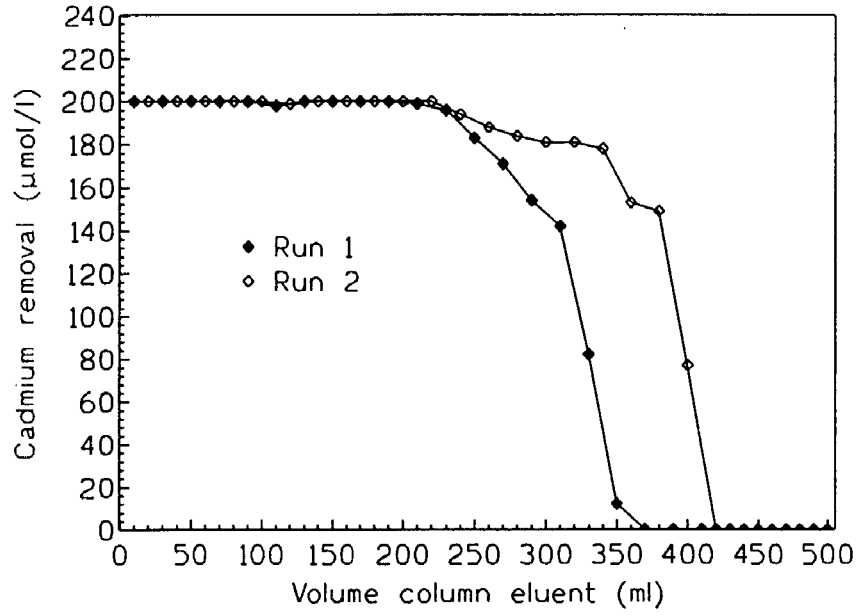

Figure 4.3: Bioaccumulation of cadmium on a 20 mℓ immobilized *Saccharomyces cerevisiae* column. The cadmium was desorbed by 0.1 M HCl, the column was reconditioned and cadmium reapplied determined reusability.

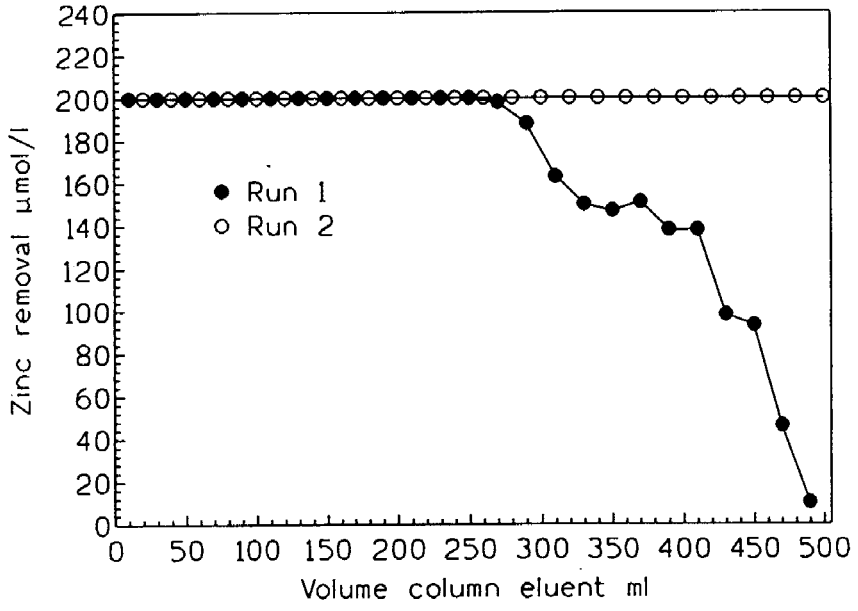

Figure 4.4: Bioaccumulation of zinc on a 20 mℓ immobilized *Saccharomyces cerevisiae* column. The zinc was desorbed by 0.1 M HCl, the column was reconditioned and zinc reapplied to determine the reusability of the biosorbent.

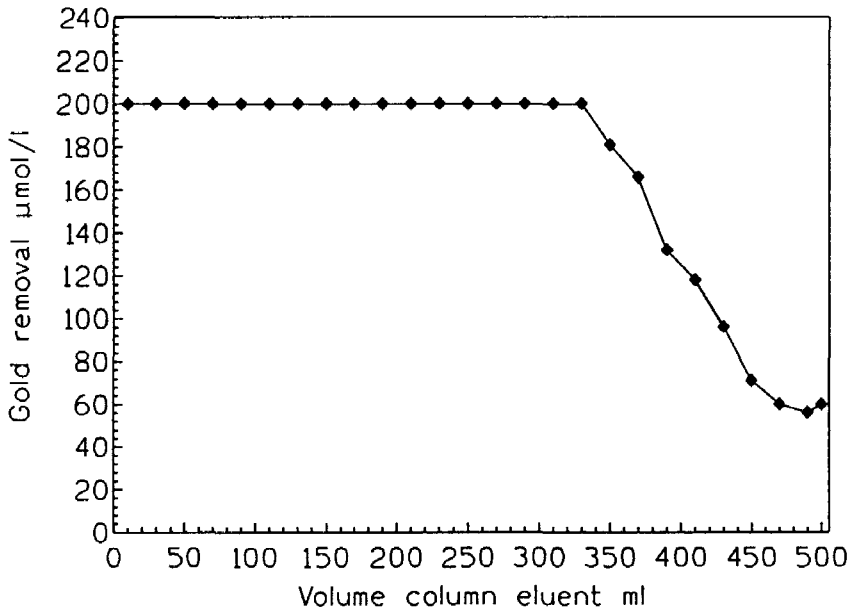

Figure 4.5: Bioaccumulation of gold on a 20 mℓ immobilized *Saccharomyces cerevisiae* column.

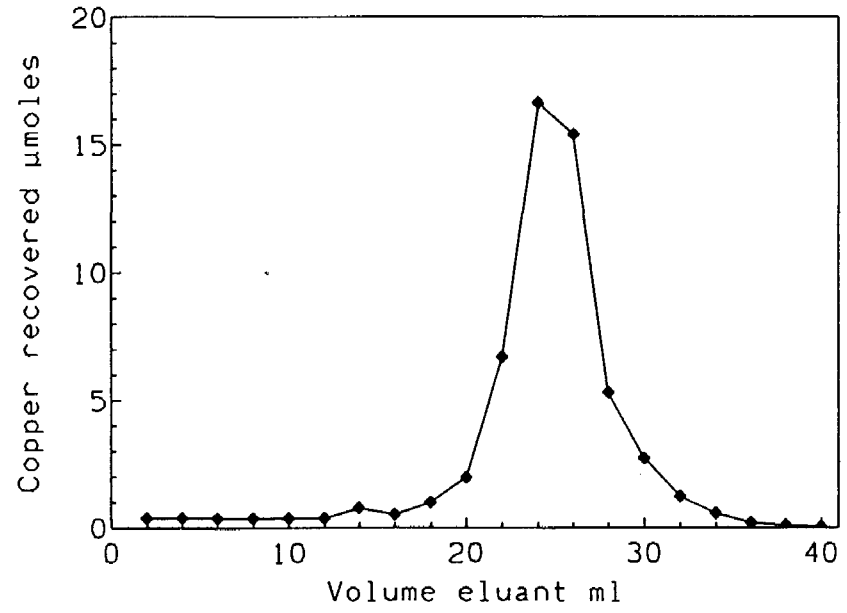

Figure 4.6 : An example of metal recovery from a 20 mℓ immobilized *Saccharomyces cerevisiae* biosorption column by elution using 40 mℓ 0.1 M HCl. This profile shows copper desorption after accumulation as shown for run 1 in Figure 4.1. Percentage bound copper recovered was 99 % of which 84 % was concentrated in 10 mℓ of the eluant.

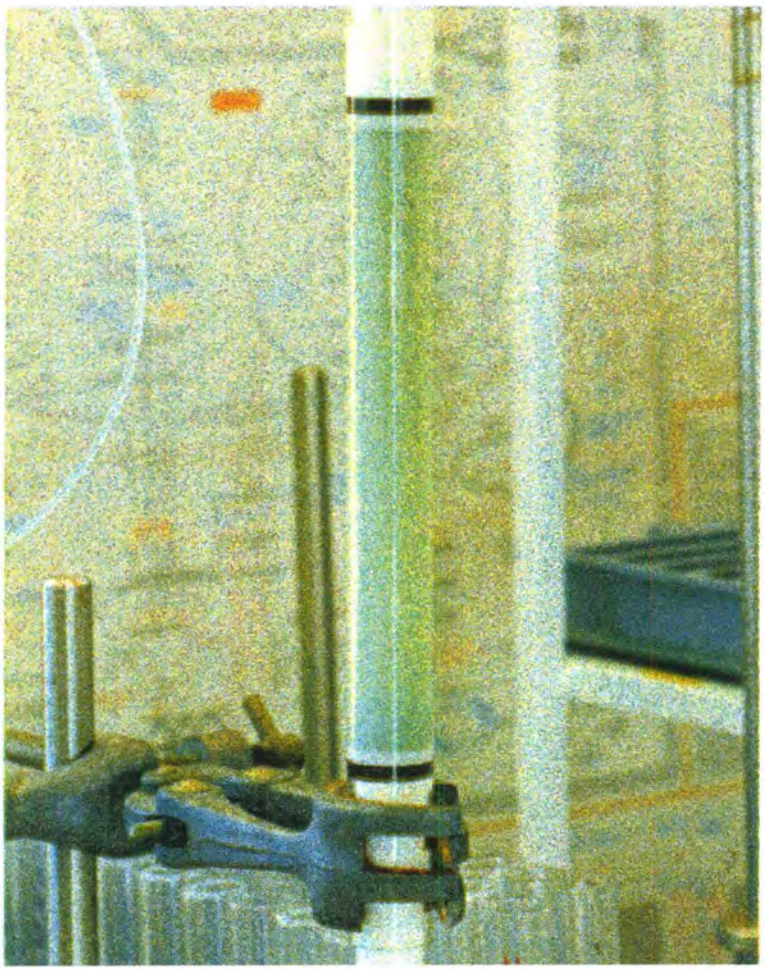

Figure 4.7 : A 20 mℓ immobilized *Saccharomyces cerevisiae* biosorption column saturated with copper. A copper solution at a initial concentration of 200 μmol/ℓ was pumped through the column in a upward-flow direction at 1 mℓ/min.

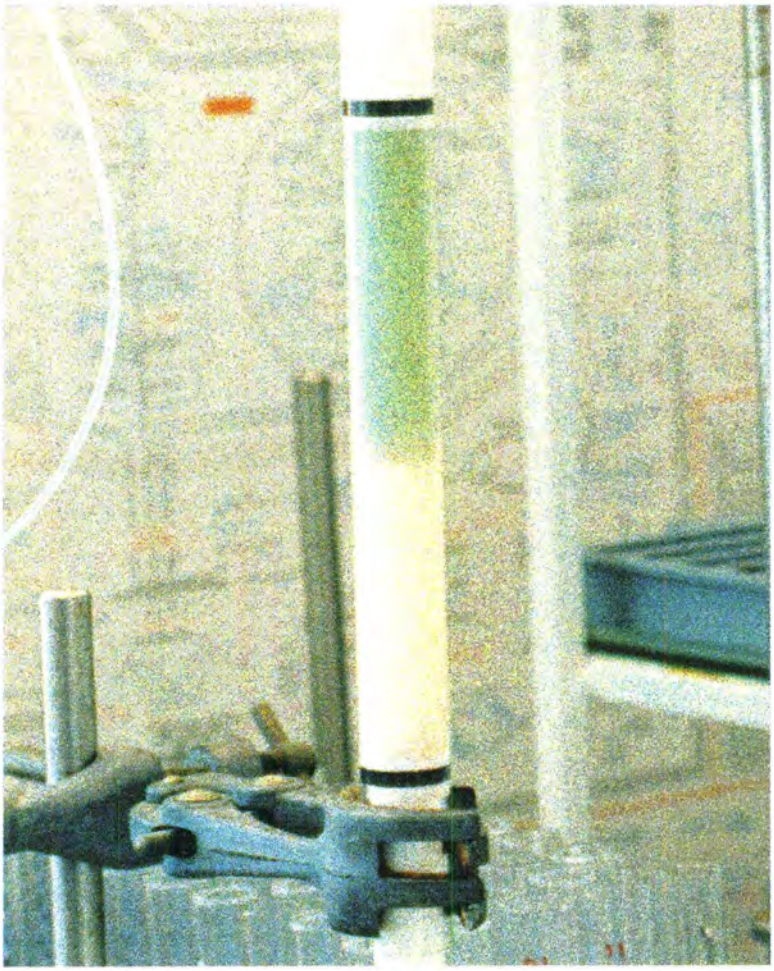

Figure 4.8 : A 20 mℓ immobilized *Saccharomyces cerevisiae* column in the process of copper desorption. The copper was desorbed by passing 0.1 M HCl through the column. Over 80 % of the accumulated copper was recovered and concentrated in 10 mℓ of the eluting solution.

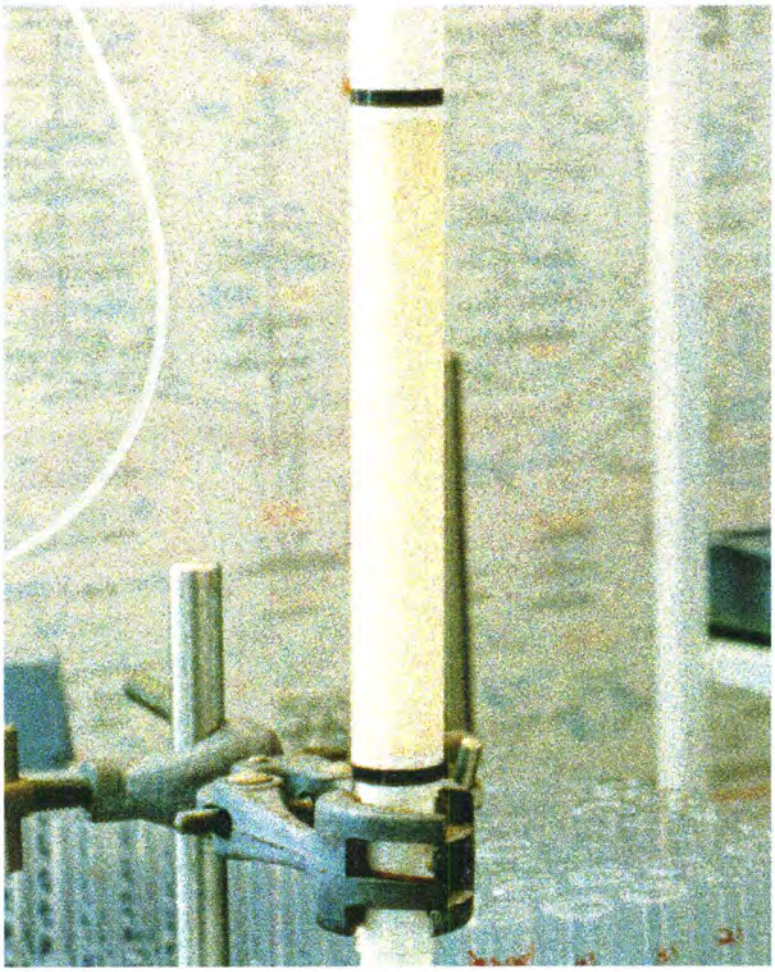

Figure 4.9 : A biosorption column post copper desorption. The column was reconditioned with 10 mℓ 0.05 M NaOH and 20 mℓ water. The column could then be reused for metal removal.

4.3.2 Selective recovery of metals from mixed metal solutions

Mixed metal solutions of two metal species were passed through two columns run in series. In the case of copper and cobalt, the first column in the series was saturated with copper after approximately 280 mℓof the mixed solution had passed through the column. At satuaration of the first column, the columns were disconnected and eluted individually with 0.1 M HCl. The metal accumulated on and recovered from each column was then determined. Examples of desorption profiles of the 2 columns are presented in Figure 4.10 and 4.11. Copper bound preferentially to cobalt on the first column at a binding ratio of 4:1, thus the metal recovered and concentrated from the first column was ≥ 75 % copper.

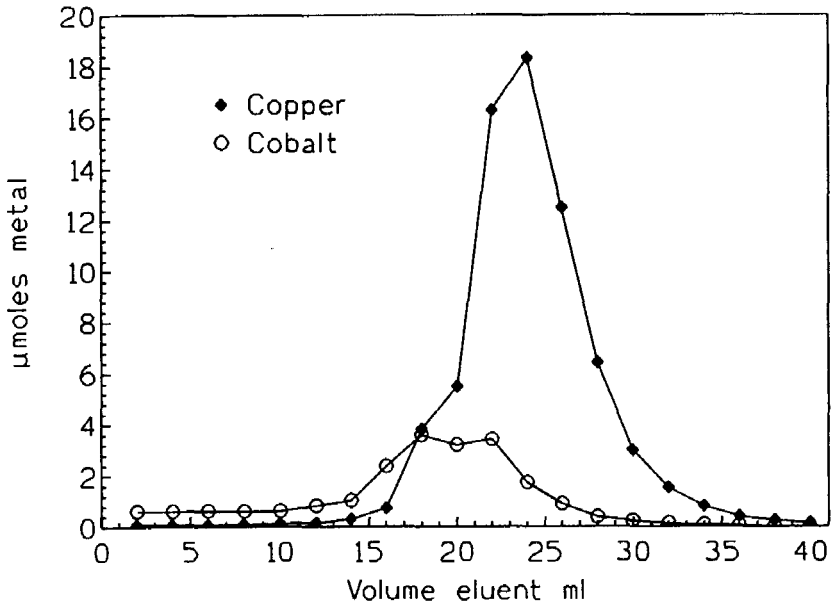

Figure 4.10: Desorption of copper and cobalt from a 20 mℓ immobilized *Saccharomyces cerevisiae* column (column 1 of 2 in series). Desorption was achieved using 0.1 M HCl. The results are expressed as μmoles metal recovered per 2mℓ eluent. The predominant metal eluted from this first column was copper.

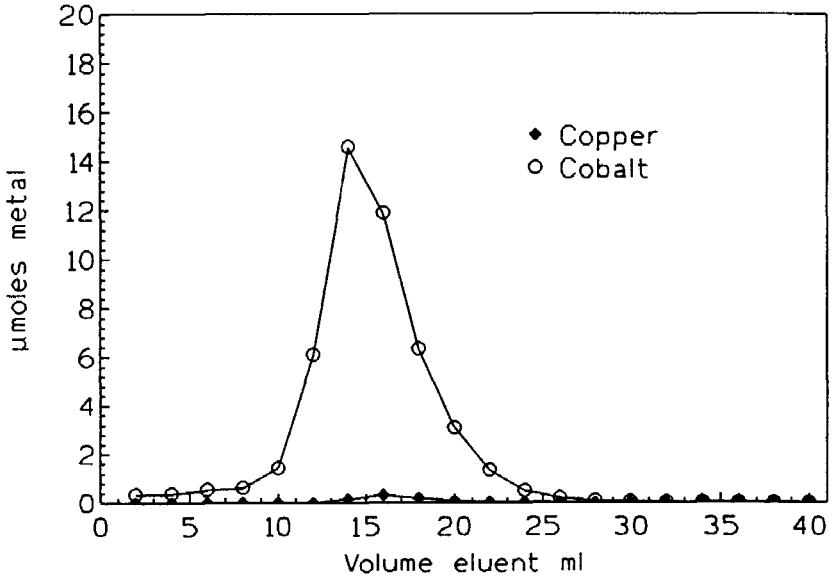

Figure 4.11: Desorption of copper and cobalt from a 20 mℓ immobilized *Saccharomyces cerevisiae* column (column 2 of 2 in series). Desorption was achieved using 0.1 M HCl. The results are expressed as µmoles metal recovered per 2mℓ eluant. The predominant metal eluted from this the second column was cobalt.

The second of the two column series contained 99 % cobalt. When the two column system was discontinued due to saturation of the first column, the second column was not saturated. This was reflected by no metals being detected in the application eluent from the second column.

Similar profiles were obtained for zinc, with copper binding preferentially to the first column a at ratio of 6:1. Although copper bound preferentially to cadmium, substantial mixing occurred and selective recovery was not achieved. The binding ratio in preference of copper to cadmium was 2:1 in the first column.

4.3.3 Repeated adsorption-desorption cycles of a biosorption column

Copper chloride was applied repeatedly for eight successive cycles to a single immobilized *S. cerevisiae* biosorption column. In between copper applications the bound metal was desorbed and the column reconditioned for repeated use. Copper bioaccumulation initially declined (cycles 2 and 3), but increased from cycle 5. The copper removal and percentage recoveries are presented in Table 4.2.

Table 4.2 : Adsorption-desorption reusability investigation. Adsorption of copper to an immobilized *S. cerevisiae* biosorption column over 8 cycles.

Run Number	Copper Adsorption μmol/g	Percentage Recovery
1	31.3	85
2	26.8	96
3	24.3	100
4	28.1	96
5	32.0	100
6	37.7	81
7	47.8	100
8	44.4	100

4.4 DISCUSSION

The metal ions were effectively removed from solution by the immobilized yeast in the fixed-bed biosorption column reactors. The biosorbent removed the metals until a saturation threshold was reached, following which the uptake declined rapidly. The majority of metals were desorbed from saturated columns and recovered from the biosorbent by eluting the column with 0.1 M HCl. The initial recovery of accumulated copper, cobalt and cadmium was 100 %. The high recovery of metal by mild acid elution concurs with passive binding to the cell walls of the *S. cerevisiae*. Bordons and Jofre (1987) proposed nickel removal was by adsorption to the outer membrane of a *Pseudomonas* bacteria due to the high recovery of accumulated metal with the addition of EDTA. Gardea-Torresdey (1996) reported that after a copper binding cycle the metal was desorbed with a few bed volumes of 0.1 M HCl. Cotoras *et al* (1993) reported the quantitative desorption of uranium, copper, cadmium and zinc at pH lower than 2. Uranium removed from 1 500 mℓ of water was completely recovered in 400 mℓ of the eluting solution. Metal recovered from the immobilized *S. cerevisiae* in this study was concentrated in small volumes. Over 80 % copper, zinc, cadmium, nickel and cobalt were

detected in 10-15 mℓ acid eluent, representing up to a 40 fold reduction from the initial volume. From a practical perspective, a high concentration factor is what is required as the volume of metal bearing solution is decreased making the effluent more manageable. High recovery of bound metal is also necessary for biosorbent reuse.

Chromium was not desorbed using 0.1 M HCl and increasing the acid concentration to 1.0 M only yielded a 34 % recovery in a relatively high volume. The inability to recover chromium by mild acid treatment could lead to selective desorption between the chromium and the other cations examined. Gold recovered was 67% from the biosorption column using an acidic $CaCl_2$ eluent. Kuyucak and Volesky (1988) eluted gold deposited on algal biomass using a mixture of 0.1 M thiourea and 0.02 M ferric ammonium sulphate solution at pH 5. As gold is a high value metal, incineration of the gold-laden biomass may be a more suitable method of recovery as it should ensure total recovery.

The columns were reconditioned and the metals reapplied to assess reusability. Bioaccumulation with the second metal application remained constant or was increased with certain metals. Cadmium, nickel and zinc exhibited substantial increases in adsorption. Cadmium was initially accumulated at a rate of 31.6 μmol/g and 37.3 μmol/g on the second application. All adsorbed cadmium was recovered by 0.1 M HCl elution and a 33% fold volume reduction was observed for both applications. Cobalt was accumulated the most successfully, with an initial 99.9% and then a 100 % removal from the applied 500 mℓ solutions. Zinc recovery from the biosorbent by acid elution was reduced after the second application, possibly a result of internalisation. Gold and chromium accumulated at initial rates of 42.2 μmol/g and 28.6 μmol/g respectively, however, because of the low recoveries of these two metals, a second cycle was not carried out.

The beneficial consequence of the adsorption-desorption cycles demonstrated the ability for continuous utilisation of the biosorption columns. Reconditioning of the biosorbent with NaOH may benefit the uptake capacity by exposing additional binding sites. An

increase in pH results in the cell surface being more negatively charged which should enhance cation adsorption (Chen and Ting, 1995).

Mixed metal solutions of two metal species were passed through two columns run in series to determine the extent of selective uptake of the cations due to different affinities for sorption binding sites on the biomass. Post metal bioaccumulation and saturation of the first of the columns, the columns were disconnected and individually washed with 0.1 M HCl. Thus the metal accumulated on each column was recovered separately. Copper bioaccumulation and recovery from the first of the columns run in series was the most efficient. The other metals investigated were displaced to the second column. Zinc was displaced by copper at a binding ratio of 6:1. Cadmium was displaced by copper, however, substantial mixing occurred and selective recovery was not achieved. Copper bound preferentially to cadmium in the first column at a ratio of 2:1. Selectively binding was clearly demonstrated between the metals copper and cobalt. Copper bound preferentially to the first column at a binding ratio of 4:1, displacing cobalt to the second column. The metal recovered and concentrated from the first column was ≥ 75 % copper. The second of the two column series bound predominantly cobalt and a 99 % pure cobalt concentrate was obtained. This investigation confirmed the potential for selective metal recovery by biosorbents, however, the system would require further optimization to obtain total metal separation.

Although copper bound preferentially to the other metals in the mixed metal biosorption columns, total copper uptake was relatively low. As an example, copper uptake from a single column, single metal situation was 27.9 μmol/g compared to cobalt uptake which was 49.7 μmol/g. Similar amounts of copper were bound to the first column in the mixed metal series. The second column was not saturated by the second metal. This suggests a higher copper binding affinity to the biosorbent, but a lower removal capacity compared to the other metals investigated.

Nakajima *et al* (1981) demonstrated the ability of *Chlorella regularis* to accumulate

metals selectively . The amount of metal cations taken up in batch reactors decreased in the order Cu » Zn ≥ Co ≥ Cd ≥ Ni. They concluded that the selectivity of *C. regularis* is due to the strength of coupling between the metals and the cell components. Norris and Kelly (1979) reported a 50 % decrease in copper uptake by free cell suspensions of *S. cerevisiae* in the presence of equimolar concentrations of nickel. The influence of competing ions is a factor which requires consideration when developing a bioremediation operation for an industrial effluent. Knowledge of the different affinities of the metals for a biosorbent can be used to maximise the efficiency of the remediation process allowing selective removal of target metals. Lewis and Kiff (1988) reported the removal of metals by immobilized *Rhizopus arrhizus* in biosorption columns to have an efficiency order of lead > copper > cadmium > zinc. The initial biosorption rate by the biomass was mostly above 80 %.

A single column was subsequently used for eight consecutive adsorption-desorption cycles. The copper uptake declined from 31.3 μmol/g to 24.2 μmol/g over 3 cycles, thereafter uptake progressively increased after each cycle to 47.8 μmol/g at cycle 7. The quantity of copper accumulated corresponded to the quantity eluted after desorption indicating complete recovery. As was observed in the batch studies in chapter 3, metal accumulation increased with repeated application and metal recovery remained high. Removal of nickel was achieved for 5 consecutive removal-regeneration cycles in a pH range of 3.8 to 5.8 using immobilized *Citrobacter sp* primed with hydrogen uranyl phosphate (Basnakova and Macaskie, 1997). Similar repeated adsorption-desorption cycles have also been reported for other metals and biosorbents by other researchers (Holan *et al*, 1993; Tsezos, 1989).

The total metal loading by the immobilized *S. cerevisiae* in this investigation were not as high as reported in some of the literature (Volesky and Prasetyo, 1994; Sharma and Forster, 1995; Kratochvil, 1995). However, the biosorption columns described in this study may be optimized to the requirements of a particular effluent and have the potential to provide an effective metal removal and recovery process. Biosorption columns may

also provide a useful and rapid method for determining optimal removal conditions, for observing selective removal and for obtaining selective recovery of metals.

4.5 CONCLUSION

The immobilized *S. cerevisiae* packed-bed biosorption columns effectively removed metals from aqueous solutions. The bioaccumulated metals were recovered by 0.1 HCl M elution in most cases. The desorption protocol utilised a minimum quantity of dilute acid and yielded a concentrated, low volume metal eluent. The ease of metal elution and recovery from the columns implied passive binding to the cell wall. The biomass was reusable. The adsorption-desorption process did not adversely affect the uptake capacity of the biosorbent and the uptake of cadmium, nickel, cobalt and zinc were enhanced on a second application. A copper solution was run on a single column through 8 cycles with no apparent decrease in biosorbent performance. The potential for selective recovery by the biosorption and elution protocol was demonstrated. The success of selective binding and recovery of metals from mixed metal solutions will be dependent on the affinity of the metals and their initial concentration in solution. The reusability of the biosorbent and the selective recovery of target metals could lead to the development of a viable, cost effective, metal bioremediation technology. The bioremediation of a mixed metal mine effluent, using immobilized *S. cerevisiae*, was investigated and is discussed in chapter 5.

CHAPTER V

THE REMOVAL OF METALS FROM MINING EFFLUENT BY IMMOBILIZED *Saccharomyces cerevisiae*

5.1 INTRODUCTION

Conventional treatment of acidic mine drainage includes; neutralization of the mine water by the addition of an alkaline chemical, aeration or introduction of a chemical oxidant to promote oxidation of reduced metal ions, and settling of insoluble substances produced by these actions (Unz and Dietz, 1986). These methods are often ineffectual and expensive, particularly when dealing with large volumes at relatively low metal concentrations ($\leq$ 100 ppm) (Volesky, 1987). Bioremediation technology can potentially provide an efficient, inexpensive alternative to these traditionally used methods. Bioremoval of metals could also be utilised to complement an established remediation operation.

Transfer of the technology from the laboratory to scaled-up operations presents a major challenge due to the unpredictable nature of different effluents and uncontrollable environmental factors. The practical reality of implementing a successful bioremediation system depends on many factors, however, possibly the most important is the ability to operate under adverse conditions (Gadd, 1989). Although the literature suggests that bioremediation of industrial waste waters is feasible, reports of successful exploitation of microorganisms are limited (Gale, 1986). Stoll and Duncan (1997) attained complete removal of copper, chromium and nickel; and $\geq$ 82 % removal of zinc and cadmium from an electroplating effluent using immobilized *S. cerevisiae* in continuous-flow reactors.

The performance of bioremediation is site specific and each effluent requires individual investigation to determine the effectiveness of the proposed process (Blackburn and Hafker, 1993). In this study bioremediation of effluents supplied from a copper, zinc and

lead mine was investigated on a fundamental level to assess the feasibility of using the immobilized *Saccharomyces cerevisiae* biosorbent. The mine's main concern was metal removal from the water so that it could be reused in metal extraction operations. The target metals in the effluent were copper, iron, zinc and lead. The pH and the range of co-contaminating waste components were potential obstacles to successful bioremediation.

5.2 MATERIALS and METHODS

5.2.1 Mine effluent

Mining effluent was supplied from Gold Fields of South Africa Ltd.

5.2.1.1 Plaatjiesvlei effluent

Effluent was obtained from Plaatjiesvlei, a wastewater catchment zone of the Black Mountain mine.

5.2.1.2 Ageing pond effluent

Following a visit to the Black Mountain mine, an ageing pond used for chemical treatment of waste water was identified as a potential site for biological process. Effluent was collected from the inlet and from the oulet of the pond.

5.2.2 pH profile

The pH of the Plaatjiesvlei effluent was sequentially adjusted with NaOH and the metal content analysed over a pH range of 2 to 6. The aging pond effluent was investigated at the collected pH.

5.2.3 Batch remediation

As described in section 2.2.4.

5.3 RESULTS

5.3.1 Metal composition of the mine effluents

5.3.1.1 Composition of the Plaatjiesvlei effluent

The composition of the Plaatjiesvlei mine effluent is presented in Table 5.1.

Table 5.1 : Composition of the Plaatjiesvlei mine effluent at pH 2.

Analysis	Concentration μmol/ℓ
Ca	22 207
Mg	4 978
Copper	21.3
Lead	7.3
Zinc	39.2
Iron	1 078
Chlorine	17 453
Sulphate	37 683

5.3.1.1 Composition of the ageing pond effluent

The composition of the ageing pond effluent is shown in Table 5.2.

Table 5.2 : The composition of the aging pond effluent at the inlet and outlet positions.

	Concentration μmol/ℓ	
Analysis	Inlet (pH 3.4)	Outlet (pH 5.6)
Copper	42.3	14.8
Lead	6.5	1.2
Zinc	24.3	4.4
Iron	987.6	3.4
Sulphate	18 917	18 917

5.3.2 pH profile of the Plaatjiesvlei effluent

The pH of the mine effluent was increased using NaOH which influenced the concentration levels of the metals levels in solution. Due to precipitation, the iron concentration was decreased from 1078 μmol/ℓ at pH 2 to 187 μmol/ℓ at pH 6, lead was decreased from 7.3 μmol/ℓ to 0.82 μmol/ℓ, copper from 21.3 μmol/ℓ to 0.6 μmol/ℓ and zinc from 39.2 to 19.3 μmol/ℓ.

5.3.3 Bioremediation of the metals from the mine effluent

5.3.3.1 Bioremediation of the Plaatjiesvlei effluent

The bioremediation process was dependent on the effluent pH. A strategy for metal removal from the effluent, using bioremediation and increasing the solution pH, was used.

At pH 2 no copper was accumulated by the biomass. At pH 3 the total removal of copper from the effluent was 44.5 % of which 32 % was accumulated by the biomass while the remaining 12.5 % was due to precipitation. At pH 4 total removal of copper was 92.5 % to which bioremediation contributed 63 %. At pH 5 and 6 the total copper removal was high, however, due to precipitation, the uptake by the biomass was negligible. Copper removal from the effluent at sequentially increased solution pH in batch reactors is shown in Figure 5.1.

At pH 2 lead removal by the biomass was 24 %. At pH 3 total removal was 42 % of which 28 % was accumulated by the biomass. At pH 4 total removal was 90 % (50 % was accumulated by the biomass). Total removal at pH 5 and 6 was 88 % and 93 % respectively and the contribution by bioaccumulation was 26 and 4 % respectively. Lead removal from the effluent is shown in Figure 5.2.

Zinc was not bioaccumulated at pH 2 or 3 and generally zinc uptake by the immobilized yeast remained low. Total removal at pH 4, 5 and 6 was 60 %, 70 % and 85 %, respectively of which only 22.5 %, 26 % and 34 %, respectively was due to

bioaccumulation. Zinc removal from the effluent is shown in Figure 5.3.

At pH 2 and 3 iron bioaccumulation was 7 % and 40 % with no precipitation. At pH ≥ 4 iron removal was mostly by precipitation. Due to the relatively low concentrations remaining in solution the uptake by the biomass was negligible. Iron removal from the effluent is shown in Figure 5.4.

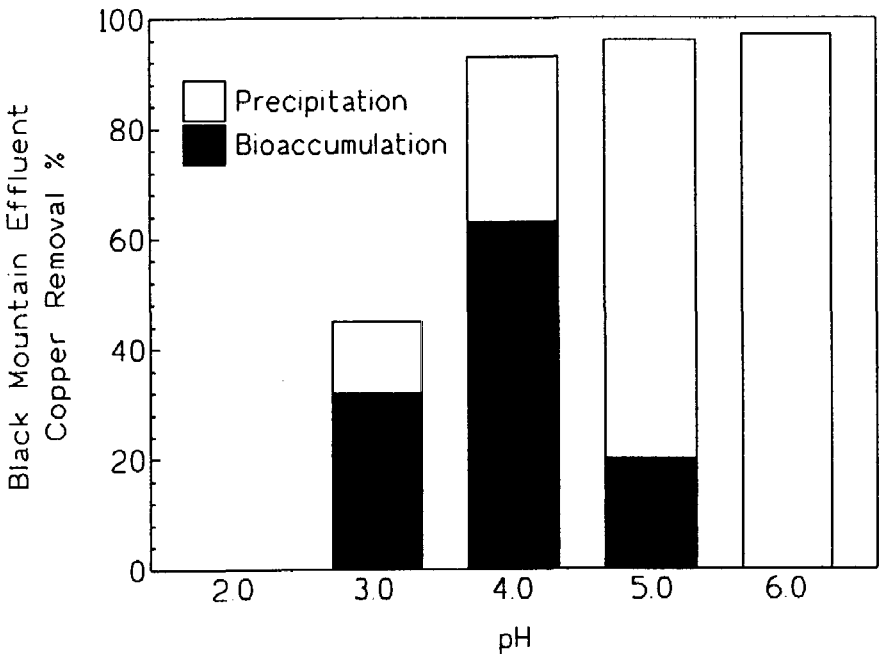

Figure 5.1: Removal of copper from the Black Mountain mine effluent by immobilized *Saccharomyces cerevisiae* in batch reactors. The initial copper concentration was 21.3 μmol/l ℓ. By increasing the effluent pH using NaOH the copper was removed from solution by □ precipitation and ■ bioaccumulation.

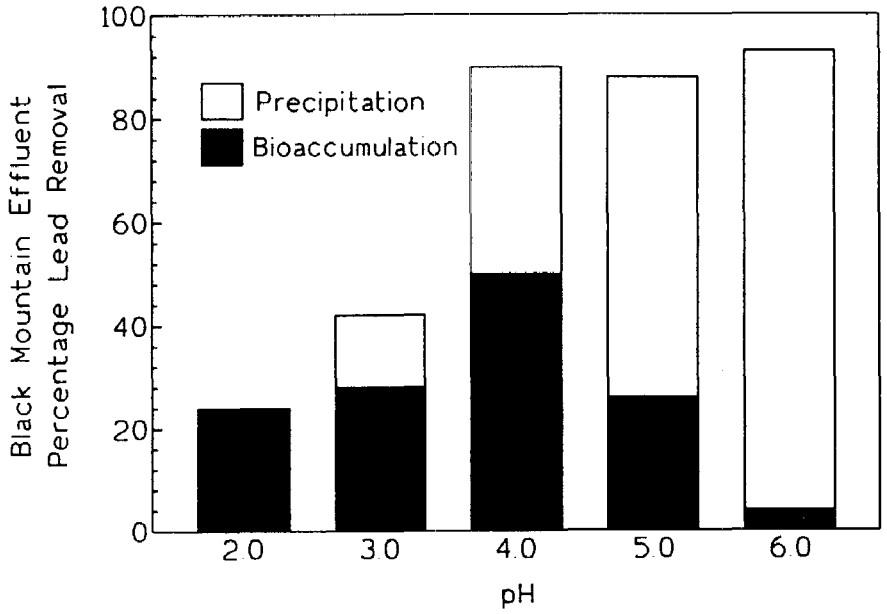

Figure 5.2: Removal of lead from the Black Mountain mine effluent by immobilized *Saccharomyces cerevisiae* in batch reactors. The initial lead concentration was 6.2 μmol/l. The effluent pH was increased using NaOH and the lead was removed from solution by □ precipitation and ■ bioaccumulation.

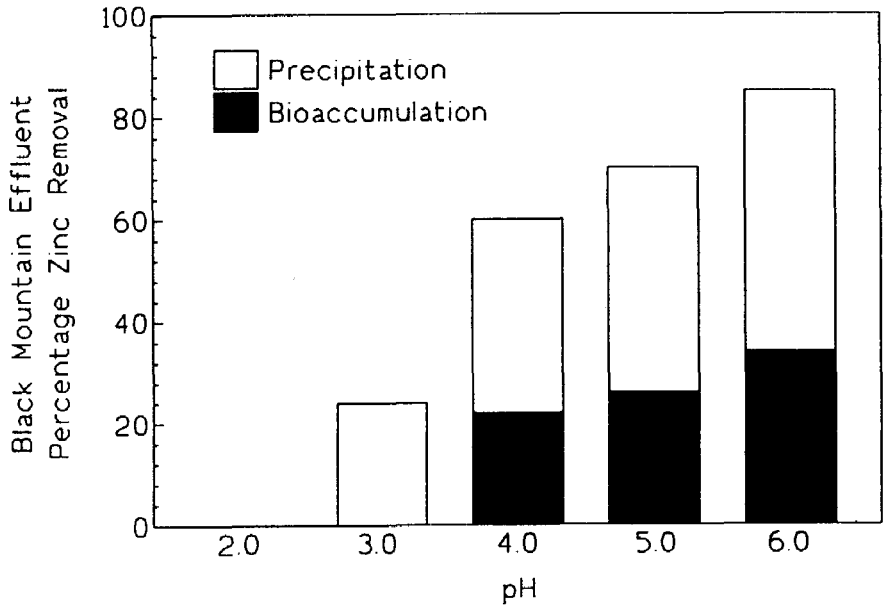

Figure 5.3: Removal of zinc from the Black Mountain mine effluent by immobilized *Saccharomyces cerevisiae* in batch reactors. The initial zinc concentration was 39.2 μmol/l. The effluent pH was increased using NaOH and the zinc was removed from solution by □ precipitation and ■ bioaccumulation.

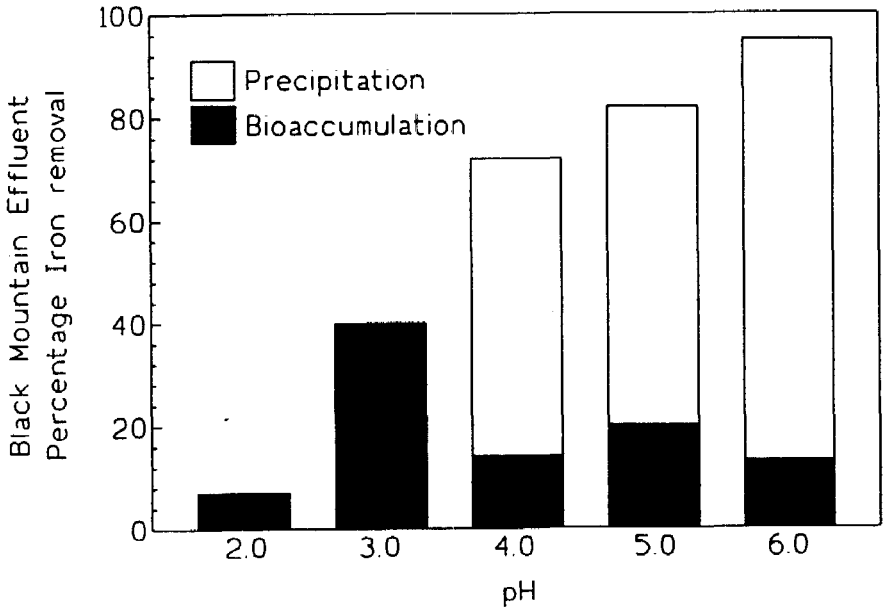

Figure 5.4: Removal of iron from the Black Mountain mine effluent by immobilized *Saccharomyces cerevisiae* in batch reactors. The initial iron concentration was 1078 μmol/l. The effluent pH was increased using NaOH and the iron was removed from solution by □ precipitation and ■ bioaccumulation.

5.3.3.2 Bioremediation of the ageing pond effluent

The bioaccumulation of metal from the effluent collected at the ageing pond outlet was as follows: copper was decreased by 64 % to 5.4 μmol/ℓ, lead by 42 % to 0.7 μmol/ℓ, zinc was decreased by 81 % to 0.8 μmol/ℓ. No iron bioaccumulation was observed. The biomass removed only a negligible amount of metal from the ageing pond inlet effluent possibly due to the relatively low pH of 3.4 and the effect of competing iron which was high at a concentration of 987.6 μmol/ℓ. Copper removal was 16 % efficient while lead, iron and zinc removal was ≤ 2 %.

5.4 DISCUSSION

The bioaccumulation of metals from the mine effluents was pH dependent. The Plaatjiesvlei effluent is complicated by the low pH which is not appropriate for cation biosorption. Bioremediation of metals from this effluent was most effective at pH 4. This

due to less competition from H^+ ions and the substantial iron precipitation at this pH. High iron concentrations have been reported to have an inhibiting effect on the uptake of coexisting metals (Galun *et al*, 1984). Because of its geological abundance iron is commonly found in mine waste waters. The ease of iron removal by precipitation supports a combination strategy of pH adjustment and bioremediation. Copper and lead were the target metals most successfully removed by bioremediation.

The Plaatjiesvlei effluent contained a multitude of contaminants such as calcium, magnesium and sulphate which may have affected metal removal. The complex nature of the effluent may have decreased the bioremediation efficiency. Lewis and Kiff (1988) reported decreased removal in effluents containing high levels of calcium and magnesium. Chong and Volesky (1995) observed that the degree of heavy metal removal from waste water depends on the multimetal competitive interactions in solution with the sorbent material. Aspects of such interactions will be dealt with in chapter VII.

The high sulphate content of the effluent is a contaminant not dealt with in this investigation, however it could provide an alternative treatment strategy. Metals can be precipitated as insoluble sulphides, which can be biologically generated by sulphate reducing bacteria from the sulphate in the effluent, as demonstrated by Hammack and Edenborn (1992).

Following a site visit, the ageing pond was identified as a potential location for a bioremediation process due to its manageable size and more favourable pH. The pond had the dimensions of 90m x 190m x 3.5m. Alkali addition at the inlet of the pond was used as the main form of treatment. The resultant sludge was pumped onto the adjacent tailings dam. The return water from the pond was reused in the extraction plant after dilution with metal free water. The major concern of the mine was to obtain metal free water for the extraction process of copper, zinc and lead. Copper and zinc contamination was reported to particularly influence the homogeneity of these metal ore extractions. Bioremediation used in conjunction with the alkaline treatment could potentially decrease

the metal contaminants to very low levels. This combined treatment strategy of the Black Mountain mine ageing pond outlet water resulted in a copper concentration of 5.4 μmol/ℓ, a lead concentration of 0.7 μmol/ℓ, zinc of 0.8 μmol/ℓ and iron of $\leq$ 3 μmol/ℓ.

5.5 CONCLUSION

The combined use of chemical precipitation and bioremediation was used to remediate a metal-laden mine effluent. Bioremediation was generally most effective at pH 4. The complexity of the effluent and its interaction with the biomaterial requires substantial fundamental and applied research before its full potential can be realized. The immobilized *Saccharomyces cerevisiae* biomass may be used to remediate metals from the effluents investigated, however, this would probably be limited to a 'polishing step' to enhance the efficiency of the chemical remediation operation. Even as a polishing procedure, one of the problems of bioremediation of mine effluents is the availability and cost of the biomass. Since it is impractical to use the baker's *S. cerevisiae* used in this study to date as a biomass because of the financial considerations, the subsequent chapters in this thesis focused on the use of a waste yeast from a brewery as a potential source of biomass for possible application in the treatment of metal contaminated effluents.

CHAPTER VI

THE REMOVAL OF METALS FROM AQUEOUS SOLUTION BY WASTE BREWER'S *Saccharomyces cerevisiae*

6.1 INTRODUCTION

The ability to remove metals from solution using *Saccharomyces cerevisiae* is well established (Blackwell *et al*, 1995; Brady and Duncan, 1994a). An important consideration in the development of a bioremediation protocol is the economic implications. *S. cerevisiae* is widely used in the fermentation industry and it is potentially available in large quantities as a waste. The use of the yeast waste product as the biosorbent material would reduce the cost of bioremediation and increase the competitiveness of the technology. However, only a few studies have focused on the use of *S. cerevisiae*, obtained directly from fermentation, as a waste product (Simmons *et al*, 1995; Singleton and Simmons, 1996). The ultimate aim of this research on the industrial waste yeast would be the development of a process which would enable utilization of the yeast as the biosorbent to remediate the effluent of a second industry at a minimal cost. In this study waste yeast was obtained from a local brewery and its metal removal characteristics investigated.

The brewing yeast was utilised in up to 6 fermentations before being discarded, the physiological status was often poor and varied from batch to batch. Mochaba *et al* (1996b) suggested that cells used continuously in fermentation had elevated levels of metals which related inversely to their physiological condition, and suggested that this phenomenon may provide a basis for vitality assays. The viability of the yeast was assayed using standard methods used in the South African brewing industry. The physiological status of the collected yeast slurries was assessed to determine a possible correlation between initial yeast viability and metal removal capabilities of the yeast. Mochaba *et al* (1996a and b) reported that lager yeast cells accumulated more metal ions on repitching,

however, less vital cells were less effective at concentrating metals.

Industrially produced waste *S. cerevisiae* cells are likely to differ in their metal biosorption capacities due to the fermentation process when compared to commercially available baker's yeast. The parameters affecting removal were thus reassessed for the waste yeast to obtain optimum biosorption conditions in batch reactors. Similar assessments were made for baker's yeast by Brady and Duncan (1994a) and for the immobilized yeast in the previous chapters in this thesis. These included biomass concentration, metal concentration, pH and time. A wash protocol for the waste *S. cerevisiae* was developed to obtain comparable and reproducible results between yeast batches. The study focussed on the removal of the metals copper, lead, zinc and iron from water as these are the metals occurring in the mine effluent under investigation (Chapter V).

S. cerevisiae cells used in batch biosorption investigations were prepared for transmission electron microscopy and X-ray analysis to confirm metal uptake by the cells. These investigations were also used to determine the metal deposition sites and to gain insight into the mechanism of metal removal by the cells. Energy dispersive X-ray analysis is reviewed by Postek *et al* (1980) and has been successfully used for metal analysis of biological material by a number of researchers (Sprey and Bochem, 1981; Jensen *et al*, 1982; Scott and Palmer, 1990; Slawson *et al*, 1992; Volesky *et al*, 1993).

6.2 MATERIALS and METHODS

6.2.1 Brewer's *Saccharomyces cerevisiae*

S. cerevisiae was supplied as a waste product by a commercial brewery. The yeast had been repitched and used in up to 6 fermentations before being discarded. The physiological condition of each batch of yeast was assessed by pH, methylene blue staining and a protease assay (Refer to Appendix 2).

6.2.2 Yeast concentration

Yeast concentration for biosorption was investigated at 0.5 mg/mℓ, 1 mg/mℓ and 10 mg/mℓ and 50 mg/mℓ. Approximately 15 mℓ of the waste yeast slurry was added to an equal volume of deionized water, vortexed and centrifuged at 500 x g for 5 min. The supernatant was discarded and 5 g of the yeast was added to 45 mℓ deionized water, the pH adjusted to 5.0 and the final volume made up to 50 mℓ to give a stock yeast solution of 100 mg/mℓ. This stock solution (10 mℓ) was diluted 1:10 to give a second stock solution of 10 mg/mℓ.

Copper sulphate stock solutions of 400.0 μmol/ℓ, 222.5 μmol/ℓ and 210.5 μmol/ℓ were prepared at pH 5. Five millilitres of the 400 μmol/ℓ copper and 5 mℓ of the yeast solution of 100 mg/mℓ were added to 100 mℓ conical flasks to give a metal concentration of 200 μmol/ℓ and a yeast concentration of 50 mg/mℓ. Nine millilitres of the 222.2 μmol/ℓ stock metal and 1 mℓ of the yeast stock solutions of 100 mg/mℓ and 10 mg/mℓ were added to 100 mℓ conical flasks to give a metal concentration of 200 μmol/ℓ and yeast concentrations of 10 mg/mℓ and 1 mg/mℓ respectively. A copper solution of 210.5 μmol/ℓ of 9.5 mℓ and 0.5 mℓ of the yeast 10 mg/mℓ solution were added to 100 mℓ conical flasks to give a metal concentration of 200 μmol/ℓ and a yeast concentration of 0.5 mg/mℓ.

In the control samples deionized water was substituted for the yeast. An incubation time of 1 h was used. Due to variations in biosorption capacities of different yeast batches and the relatively low metal uptake, a yeast conditioning protocol was developed.

6.2.3 Yeast conditioning

The cells were centrifuged for 10 min at 3 000 x g in a Beckman model J2-21 refrigerated centrifuge and the wort supernatant discarded. The pelleted cells were prepared for metal removal studies as follows: 7 g of cells were added to 20 mℓ 1.0 M HCl, vortexed and centrifuged at 500 x g for 5 min. The supernatant was discarded and the cells washed with 20 mℓ 0.25 M NaOH. The cells were finally washed in deionized water. Five grams of the conditioned cells were added to 45 mℓ deionized water, the pH adjusted to 5.0 and

the final volume made up to 50 mℓ to give a stock yeast solution of 100 mg/mℓ. The yeast was prepared immediately before use and stirred using a magnetic stirrer to maintain a consistent solution.

Metal removal by unconditioned and conditioned yeast was investigated in batch reactors. Yeast volumes, of 1 mℓ from 100 mg/mℓ stock solutions, were added to 9 mℓ 222.2 μmol/ℓ copper solutions and mixed for 1 h. The reaction mixtures were centrifuged for 5 min at 500 x g and the supernatant analysed for residual metal.

6.2.4 Adsorption time profiles

Stock single metal solutions of copper, iron and zinc sulphates and lead nitrate (purchased from Saarchem) of 222.2 μmol/ℓ were prepared in deionized water. Nine millilitres of the stock metal and 1 mℓ of the yeast solution was added to 100 mℓ conical flasks to give a metal concentration of 200 μmol/ℓ and a yeast concentration of 10 mg/mℓ. In the control samples 1 mℓ deionized water substituted the yeast. The contact times for incubation were 15, 30, 60, 120 and 360 min with shaking at ambient temperature. The reaction mixtures were then centrifuged for 5 min at 500 x g and the supernatant analysed for residual metal.

6.2.5 pH profiles

The pH of the metal solutions were adjusted using NaOH and HCl and the removal by yeast investigated over a pH range of 2.0 to 6.0 in batch reactors for 30 minutes. The methods of incubation and analysis were described in 6.2.4.

6.2.6 Equilibrium profiles

Metal removal by the waste biosorbent was investigated over the concentration range of 100, 200, 300, 400 and 500 μmol/ℓ. Incubation in batch reactors was as described in 6.2.3.

The determinations were repeated 5 times per parameter and each experiment repeated

up to 3 times. Appropriate controls were examined throughout the biosorption experiments. The uptake of metal by the yeast was determined as follows:

$q = V (C_i - C_f) / W$, where

q ~ uptake of metal in μmoles per gram of yeast

V ~ volume of the reaction mixture

C_i ~ initial metal concentration (concentration of control samples)

C_f ~ final metal concentration

W ~ mass of the biosorbent (de Carvalho *et al*, 1995).

6.2.7 Transmission Electron Microscopy

Transmission electron microscopy (TEM) was used to observe the cells used in batch reactor metal removal studies. Cells were incubated in batch reactors containing 200 μmoles/ℓ of metal solution for 30 min. Control cells were washed as described in 6.2.2.

The cell samples were fixed in a 2.5 % glutaraldehyde solution in a 5 mM PIPES buffer for 12 h, followed by two 10 min washes in 5 mM PIPES buffer. The cells were dehydrated in a series of ethanol solutions of 30 to 100%. Infiltration and embedding of samples was initiated by two propylene oxide washes. The cells were placed in eppendorf tubes containing a 75:25 ratio of propylene oxide to TAAB 812 Araldite CY 212 resin for 24 h. The propylene oxide resin mixture was decanted and replaced with a 50:50 mixture for 24 h and the process repeated for a 25:75 mixture. The cells were then placed in 100 % resin for 24 h at 4° C. The specimens were embedded in a TAAB 812 Araldite CY 212 resin at 60°C for 36 h.

A RMC MT7 ultramicrotome with glass knives was used for trimming and sectioning the polymerized resin blocks. Sections of 320 nm were cut and mounted on 300 mesh grids. Samples exposed to copper were mounted on gold grids and the lead, iron and zinc samples mounted on copper grids. Samples were not stained. Transmission electron micrographs were taken using a JOEL JEM 100 CX electron microscope operated at an acceleration voltage of 80 kV.

6.2.8 Energy Dispersive X-rays Analysis

Specimens prepared for TEM were also used for energy dispersive X-ray analysis (EDXA). EDXA spectra of intracellular elements were generated using a Philips EM 420 transmission electron microscope coupled to a EDAX DX-4 X-ray microanalysis system at the University of Port Elizabeth. The accelerating voltage of the TEM was 120 kV. Spectra were obtained from the cell wall and cytoplasm of the cells.

6.3 RESULTS

6.3.1 Yeast physiology

Yeast pH, methylene blue stain and a protease assay were used as indicators of cell physiology. The waste yeast obtained from the brewery differed from batch to batch and was generally in a poor condition. The pH of different batches of yeast were mostly above 5.5. The methylene blue viability stain of cells, from different batches, gave observed viabilities of between 50 and 85 %. The protease assay gave absorbance differences of 0.02 and 0.04 indicating varying degrees of cell lysis. The yeast cells were collected in a stationary phase and could be stored at 4° C for up to 3 weeks without a further significant loss in viability or deterioration in the yeasts ability to remove metals from solution.

6.3.2 Yeast concentration and metal removal

The effect of unconditioned yeast concentration on copper removal is shown in Table 6.1.

Table 6.1 : The effect of yeast concentration on the removal of copper from solution.

Yeast concentration (mg/mℓ)	Removal (%)	Accumulation (μmol/g)
0.5	13.0 ± 0.56[a]	51.8 ± 2.42[a]
1.0	16.2 ± 1.31[a]	32.4 ± 2.81[a]
10.0	53.5 ± 0.64[a]	10.7 ± 0.14[a]
50.0	63.9 ± 0.85[a]	2.6 ± 0.05[a]

a - Mean ± SD of 5 determinations

The percentage of copper removed from solution increased with increasing yeast concentrations. However, accumulation per gram of yeast decreased with increasing yeast concentrations.

6.3.3 Effect of yeast conditioning on metal removal

Unconditioned yeast slurries were washed with an equal volume of water and centrifuged to remove alcohol. Table 6.1 shows an example of the variation of metal removal by waste yeast collected at different intervals and the effect of conditioning.

Table 6.2: The percentage removal of copper from solution by conditioned and unconditioned brewer's yeast from two separated batches. The initial copper concentration was 200 μmol/ℓ.

A. Yeast collected after 5 lager fermentations		B. Batch collected after 3 lager fermentations	
Treatment	Removal (%)	Treatment	Removal (%)
Unconditioned	50.8 ± 1.2[a]	Unconditioned	35.5 ± 0.9[a]
Conditioned	67.1 ± 1.1[a]	Conditioned	70.15 ± 0.6[a]

a - Mean ± SD of 5 determinations

6.3.4 Effect of time on metal removal

The ability of waste brewer's yeast to remove the metals; copper, lead, zinc and iron from single metal ion solutions of 200 μmol/ℓ was investigated at the natural pH of the solution. The yeast concentration was 10mg/mℓ. A copper biosorption time profile is presented in Figure 6.1. The removal of copper at its natural pH of 5.5 was 14.7 μmol/g after 15 min incubation, increasing slightly to 15.1 μmol/g at 60 min and 15.5 μmol/g at 360 min. Time removal investigations of lead and zinc gave very similar profiles to copper. Lead removal at natural pH 5.37 was 13.1 μmol/g at 15 min and remained constant up to 360 min. Zinc removal at natural pH 5.78 was low and increased from 6.1 μmol/g at 15 min to 6.5 μmol/g at 120 min. Iron removal at pH 5.5 was relatively low at 6.9 μmol/g after 15 min and increased to 7.9 μmol/g at 120 min and 9.5 μmol/g after an incubation time of 360 min. An iron biosorption time profile is presented in Figure 6.2.

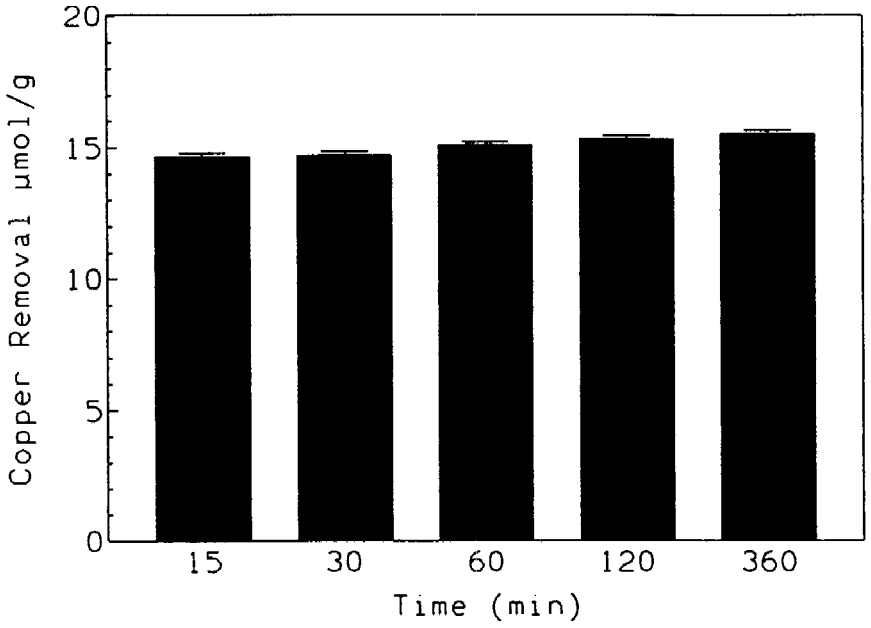

Figure 6.1: The effect of time on the removal of copper from solution in a batch reactor by a free cell suspension of conditioned brewer's *Saccharomyces cerevisiae* (n = 5, ± SD). The initial metal concentration was 200 μmol/ℓ and the yeast concentration 10 mg/mℓ.

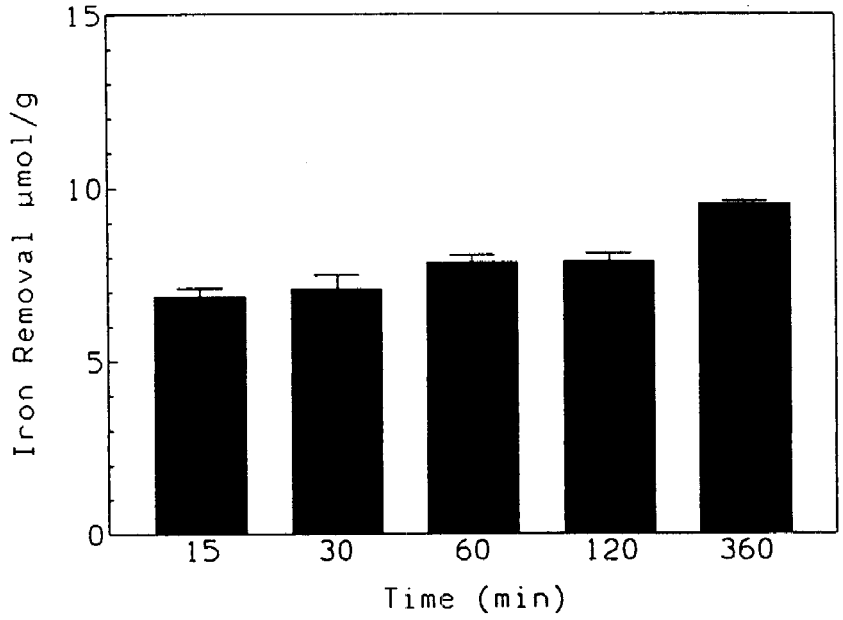

Figure 6.2: The effect of time on the removal of iron from solution in a batch reactor by a free cell suspension of conditioned brewer's *Saccharomyces cerevisiae* (n = 5, ± SD). The initial metal concentration was 200 μmol/ℓ and the yeast concentration 10 mg/mℓ.

6.3.5 Effect of pH on metal removal

The pH profiles ranged between pH 2.0 and 6.0 with the pH of the metal solutions adjusted prior to contact with the yeast. A typical pH profile of metal removal by the waste yeast is presented in Figure 6.3. The removal of the metals investigated was highest at pH 4 and above. Copper removal at pH 6.0 was 14.8 μmol/g and decreased to 7.1 μmol/g at pH 3.0 and to 4.4 μmol/g at pH 2. Lead removal decreased from 15.9 μmol/g at pH 5.0 to 5.8 μmol/g at pH 3.0. Removal was negligible at an initial solution pH 2.0 at 1.0 μmol/g. Zinc removal was 7.6 μmol/g at pH 6.0 and decreased to 2.2 and 1.9 μmol/g at pH 3.0 and 2.0 respectively. Iron removal decreased from 9.4 μmol/g at pH 5.0 to 5.2 μmol/g at pH 2.0.

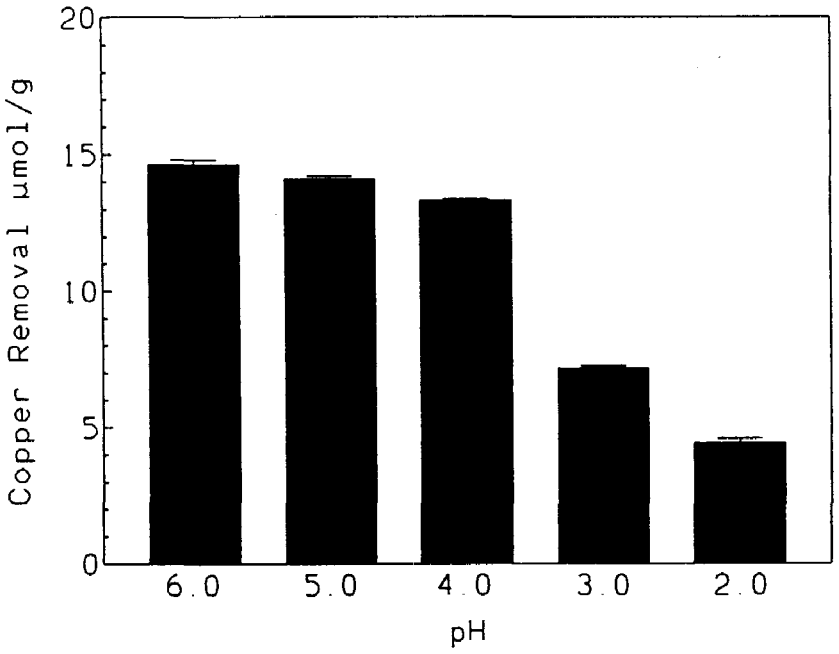

Figure 6.3: The effect of initial solution pH on the removal of copper from solution in a batch reactor by a free cell suspension of conditioned brewer's *Saccharomyces cerevisiae* (n = 5, ± SD). The initial metal concentration was 200 μmol/ℓ and the yeast concentration 10 mg/mℓ.

6.3.6 Biosorption isotherms

Binding isotherms for copper, lead, zinc and iron were generated over the initial concentration range of 100 to 500 μmol/ℓ. Maximum removal capacities and dissociation constants were determined by linear transformation of Michaelis-Menten isotherms. The maximum removal (B_{max}) and Kd by the waste yeast for copper was 25.4 μmol/g and 262.0 μmol/ℓ respectively. The copper sorption isotherm and a Hanes-Woolf linear plot are shown in Figure 6.4. The B_{max} and Kd for lead removal was 19.9 μmol/g and 111.2 μmol/ℓ respectively (Refer to Figure 6.5), the zinc B_{max} and Kd were 12.5 μmol/g and 92.9 μmol/ℓ respectively (Refer to Figure 6.6) and the iron B_{max} and Kd were 15.6 μmol/g and 177.0 μmol/ℓ respectively (Refer to Figure 6.7).

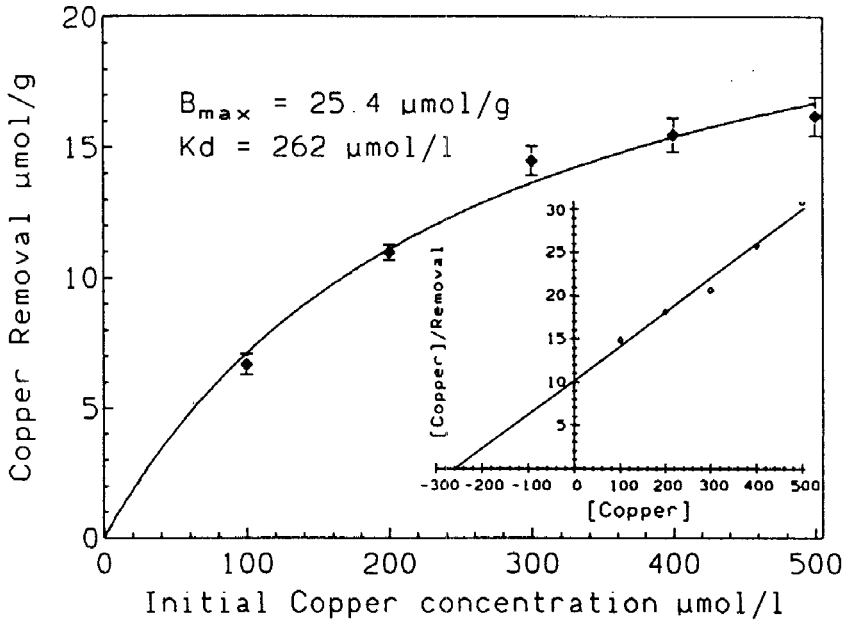

Figure 6.4: A sorption isotherm of initial copper ion concentrations and the removal rates by brewer's *Saccharomyces cerevisiae* (n = 6, ± SD). The incubation time was 30 min, the pH 5.0 and the yeast concentration 10 mg/mℓ. The inset graph is a Hanes-Woolf plot. Kd and B_{max} were determined from the linear plot of the isotherm as 262.0 μmol/ℓ and 25.4 μmol/g respectively.

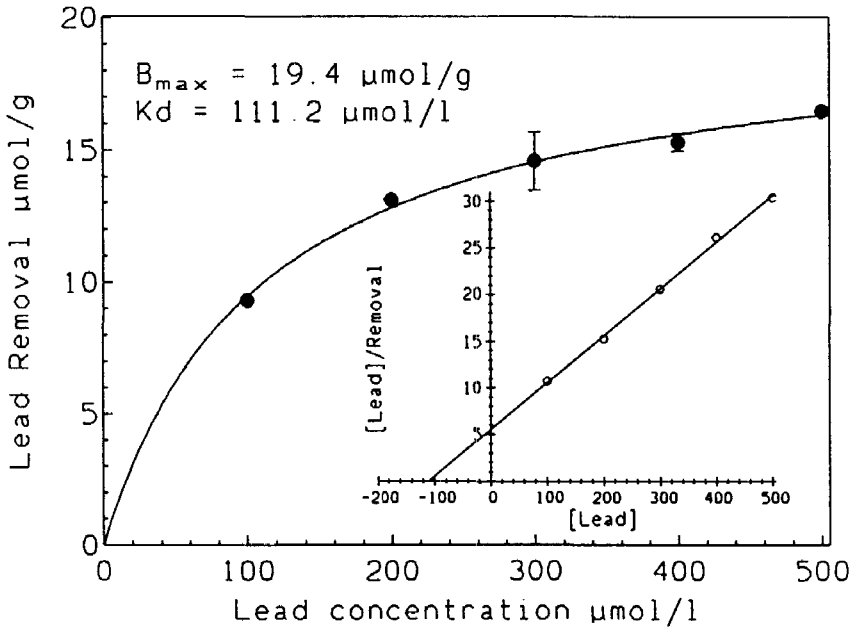

Figure 6.5: A sorption isotherm of initial lead ion concentrations and the removal rates by brewer's *Saccharomyces cerevisiae* (n = 6, ± SD). The incubation time was 30 min, the pH 5.0 and the yeast concentration 10 mg/mℓ. The inset graph is a Hanes-Woolf plot. Kd and B_{max} were determined from the linear plot of the isotherm as 111.2 μmol/ℓ and 19.4 μmol/g respectively.

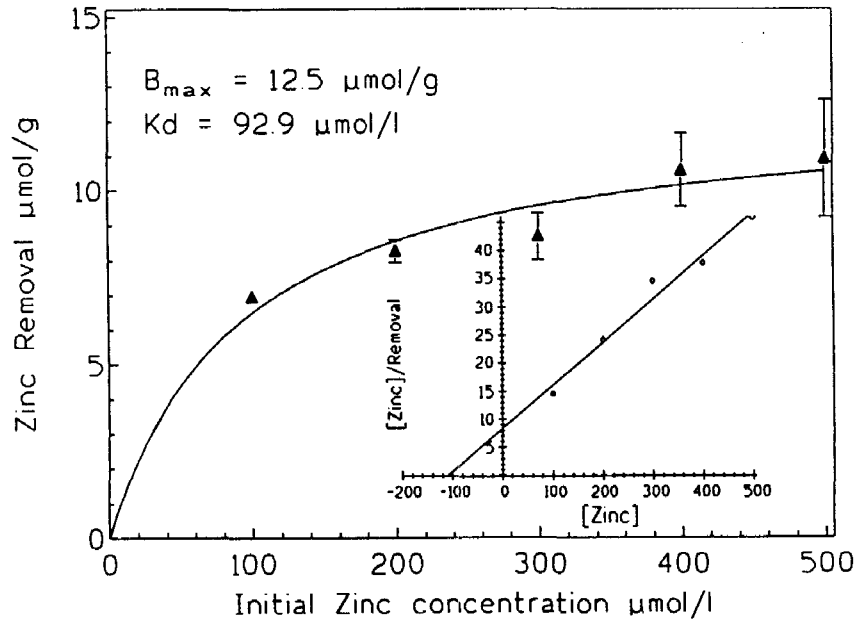

Figure 6.6: A sorption isotherm of initial zinc ion concentrations and the removal rates by brewer's *Saccharomyces cerevisiae* (n = 6, ± SD). The incubation time was 30 min, pH 5.0 and the yeast concentration 10 mg/mℓ. The inset graph is a Hanes-Woolf plot. Kd and B_{max} were determined from the linear plot of the isotherm as 92.9 μmol/ℓ and 12.5 μmol/g respectively.

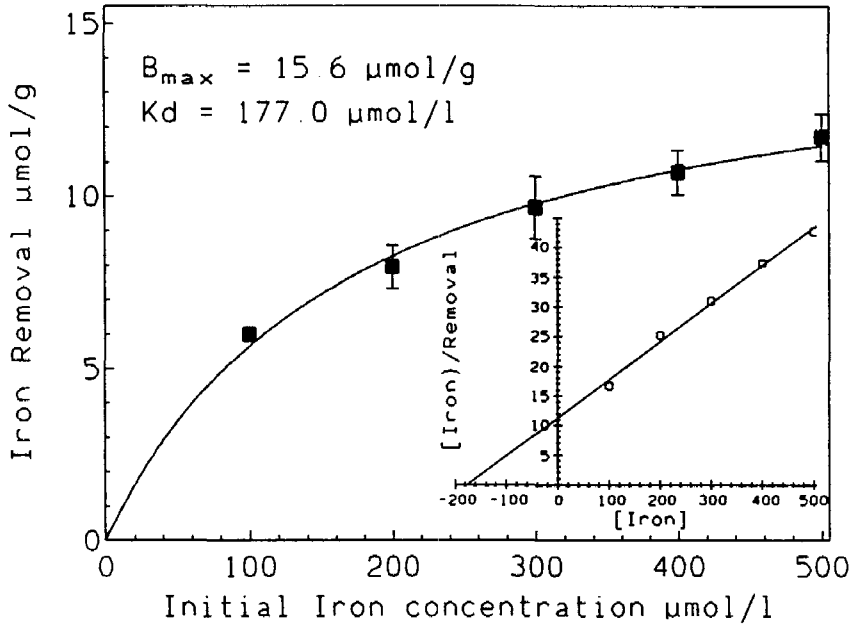

Figure 6.7: A sorption isotherm of initial iron ion concentrations and the removal rates by brewer's *Saccharomyces cerevisiae* (n = 6, ± SD). The incubation time was 30 min, pH 5.0 and the yeast concentration 10 mg/mℓ. The inset graph is a Hanes-Woolf plot. Kd and B_{max} were determined from the linear plot of the isotherm as 177.0 µmol/ℓ and 15.6 µmol/g respectively.

6.3.7 Transmission electron micrographs and EDXA spectra

No internal cellular structures were observed in the conditioned control *S. cerevisiae* cells examined under the transmission electron microscope due to the samples not being stained in the preparation process. These control cells were characterized by clear cell wall regions. A control cell is shown in Figure 6.8. A background EDXA spectrum of a clear section of resin is presented in figure 6.9. The background spectra only gave a carbon peak and copper peaks due to the copper grid. The corresponding EDXA of a conditioned cell cytoplasm detected phosphorus and chlorine. The control cell spectrum is shown in Figure 6.10. The gold peaks were due to the gold grid. Similar EDXA spectra of conditioned control cells mounted on copper grids were obtained (spectra not shown).

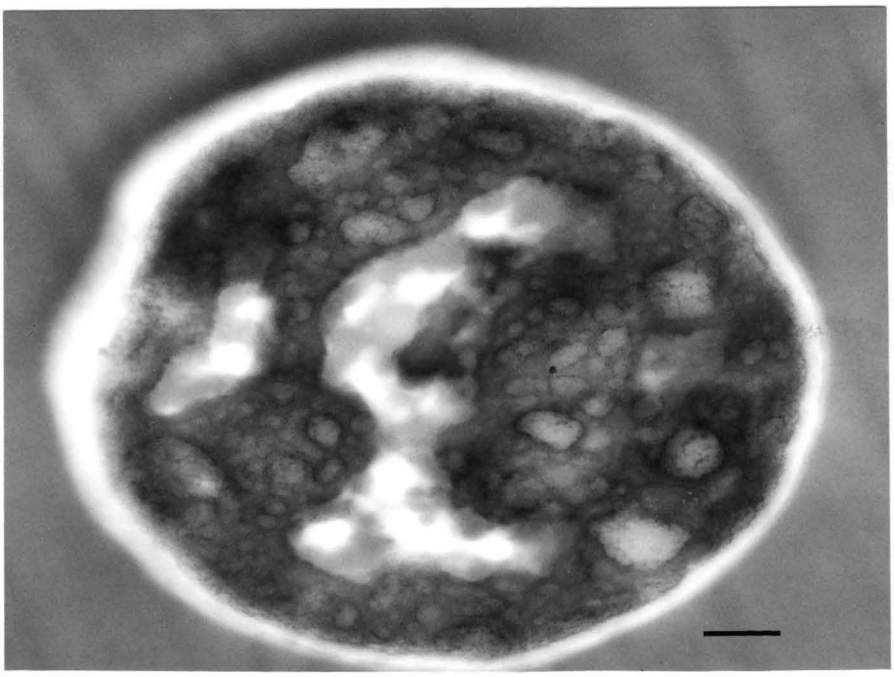

Figure 6.8: A TEM micrograph of a control *S. cerevisiae* cell after conditioning with HCl and NaOH. Apparent in the controls was the clear cell wall region. Scale bar = 0.5μm.

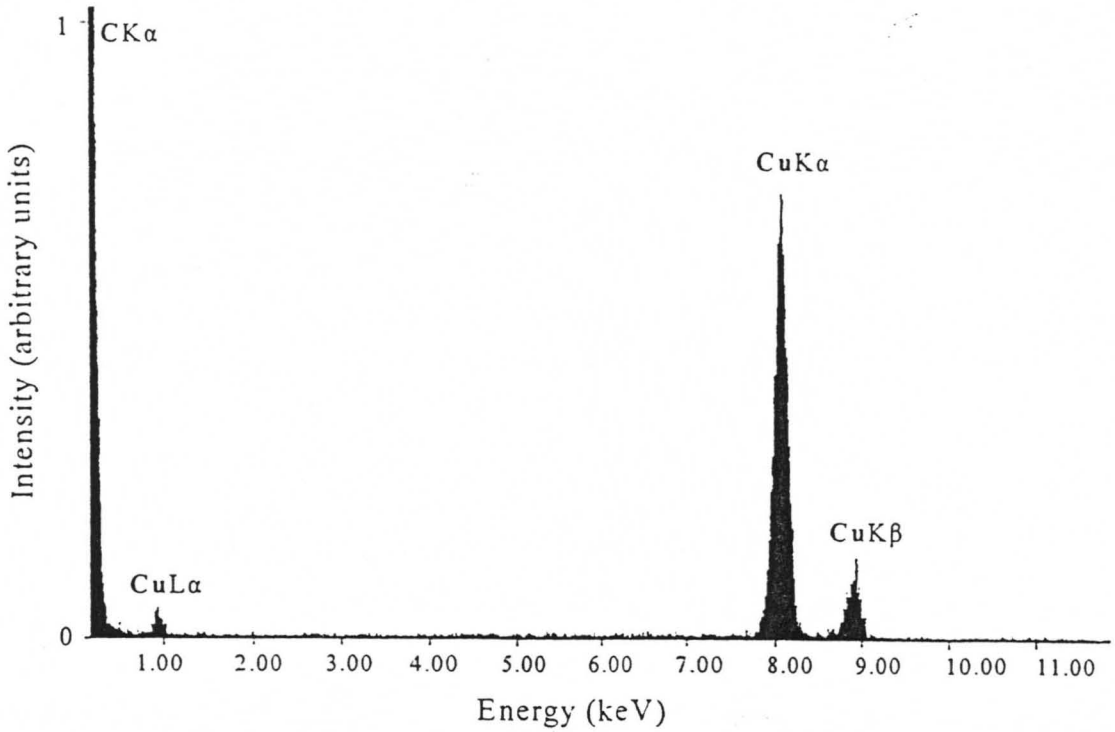

Figure 6.9: A background X-ray spectrum of the supporting resin to determine its elemental contribution. Carbon and copper peaks were identifiable. The copper peaks are due to the supporting copper grids.

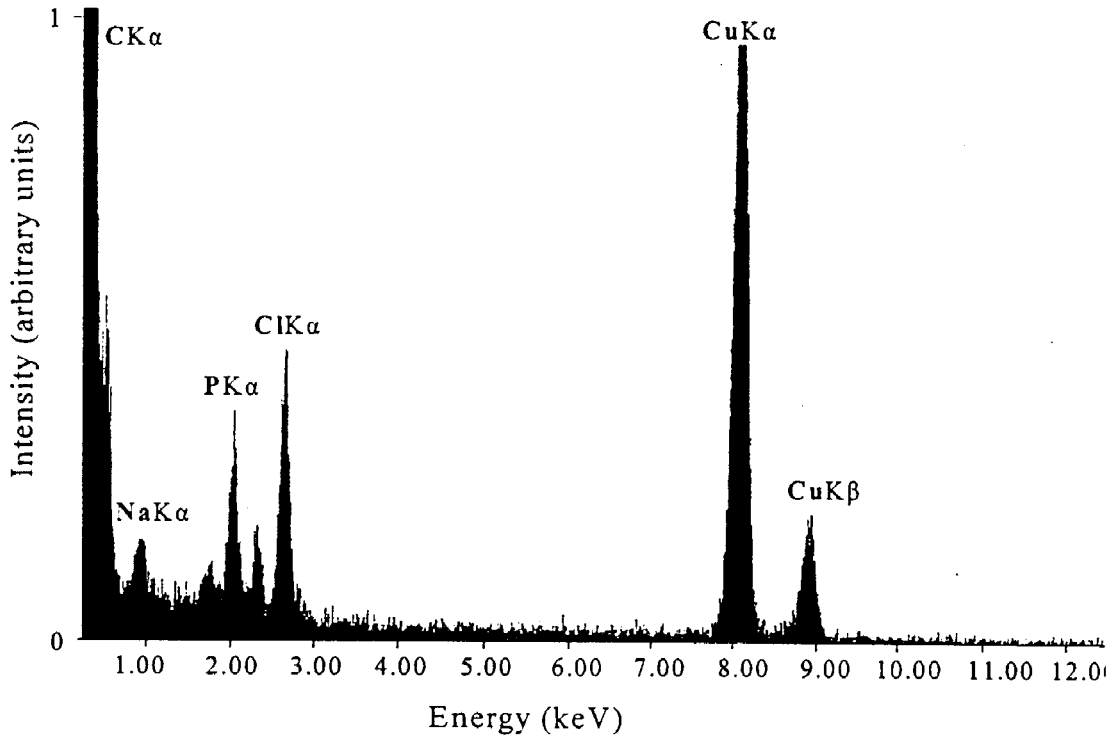

Figure 6.10: Spectrum obtained from the cytoplasm of a control cell of *Saccharomyces cerevisiae* not exposed to metal. The control cells were conditioned in HCl and NaOH. The major identifiable peaks were P (2.01 keV) and Cl (2.63). The Cu peaks are due to the supporting copper grids.

A gluteraldehyde fixed *S. cerevisiae* cell exposed to 200 μmol/ℓ copper sulphate solution is shown in Figure 6.11. The ultrastructures, although still undefined, are more prominent in these cells exposed to the copper solution. Electron dense inclusions occurred throughout the cytoplasm and were not localised in one region. Energy-dispersive X-ray analysis of these inclusions confirmed the presence of copper in the cytoplasm . The EDXA spectrum is shown in Figure 6.12. The cell wall regions of the cells which were clear in the controls, were significantly more electron dense in these cells. The EDXA confirmed the presence of copper. The spectrum is shown in Figure 6.13.

A micrograph of a *S. cerevisiae* cell exposed to lead is presented in Figure 6.14. As in the controls, no clear internal organelles could be defined. The lead was observed in lead exposed cells as fine electron dense specks throughout the cell. X-ray analysis confirmed the presence of lead in these regions. The cytoplasm and cell wall spectra are presented in Figure 6.15 and 6.16 respectively.

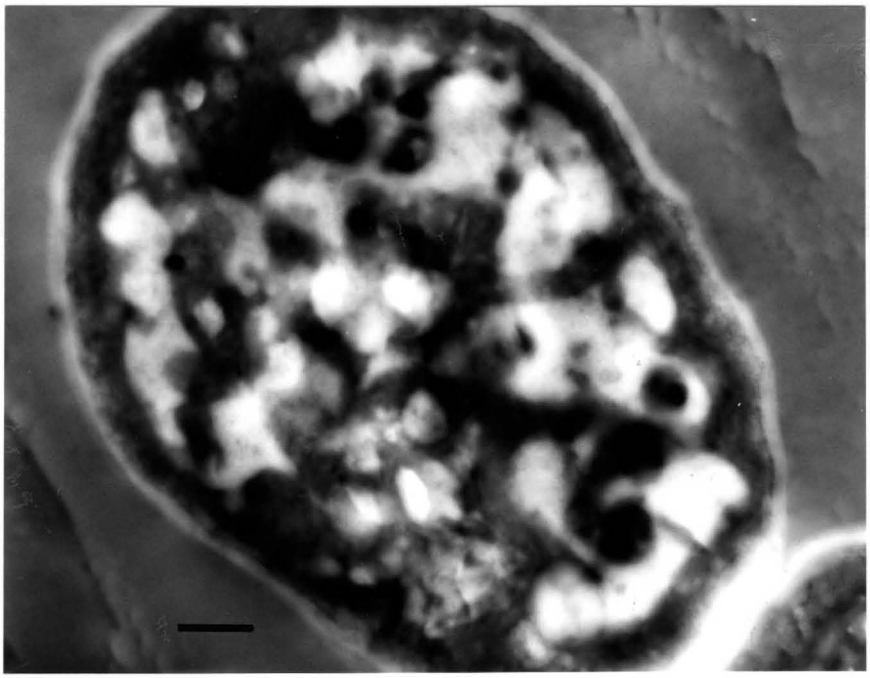

Figure 6.11: A TEM micrograph of a cross section of a *S. cerevisiae* cell after exposure to a copper solution. Scale bar = 0.5μm.

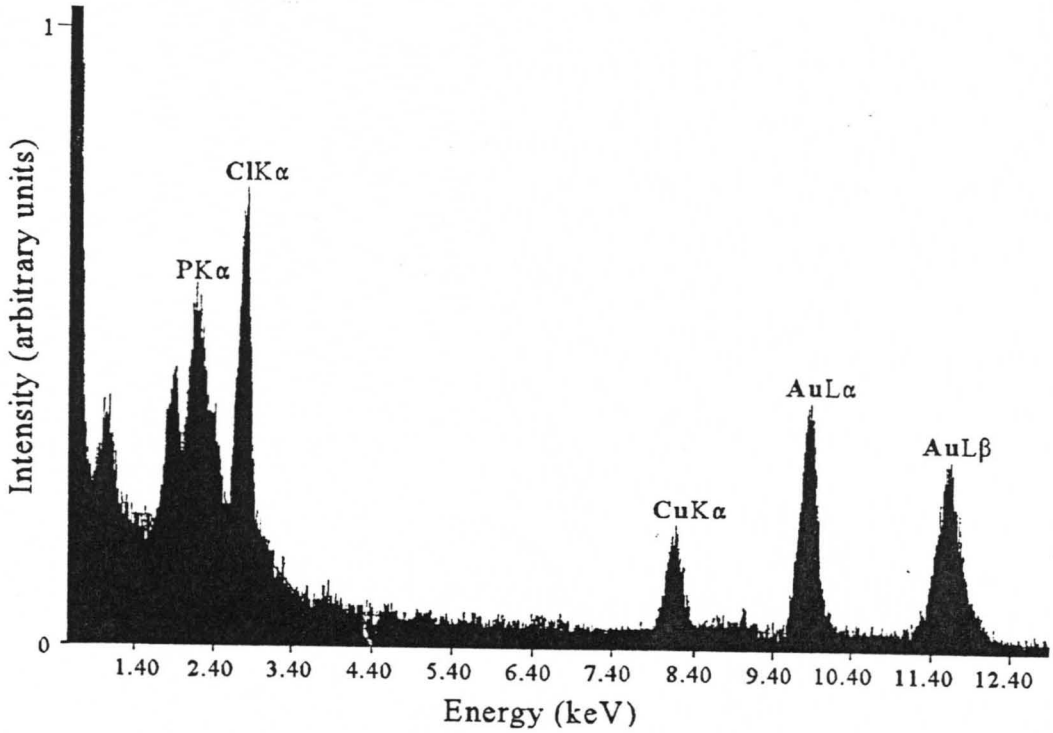

Figure 6.12: Spectrum obtained from the cytoplasm of a cell of *Saccharomyces cerevisiae* exposed to 200μmoles/ℓ of copper. Major identifiable peaks are P (2.01 keV), Cl (2.63 keV) and Cu (8.08 keV). The Au peaks are due to the supporting gold grids.

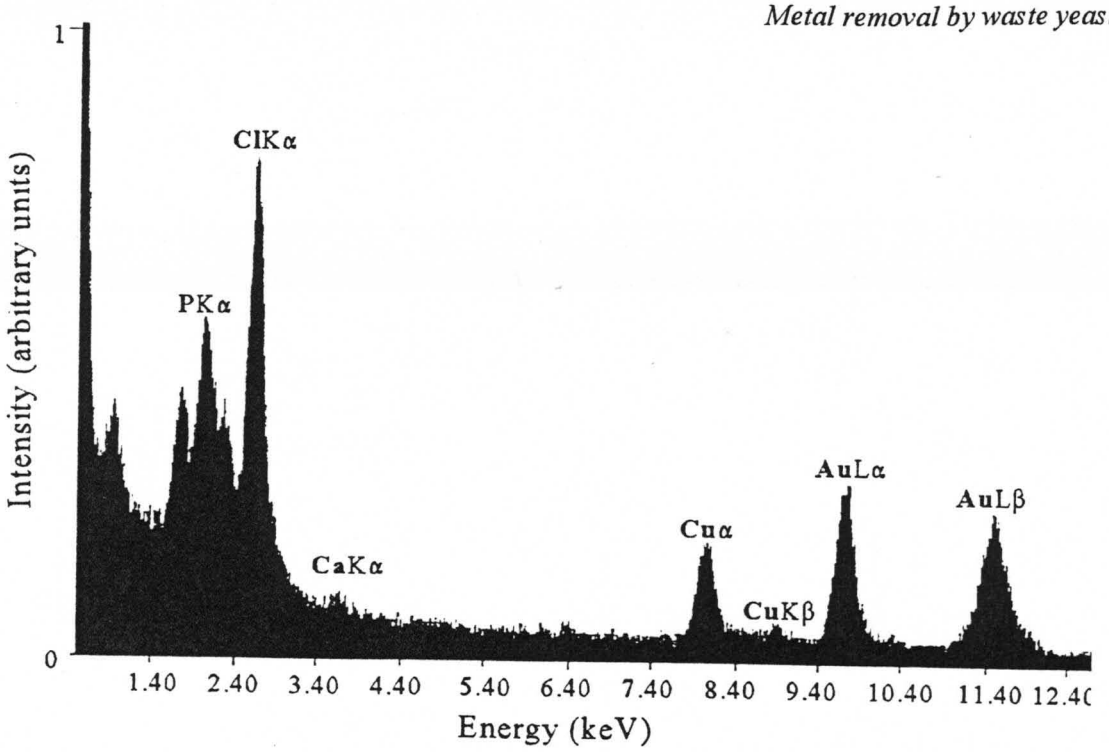

Figure 6.13: Spectrum obtained from the margin (wall) of a cell of *Saccharomyces cerevisiae* exposed to 200μmoles/ℓ of copper. Major identifiable peaks are P (2.01 keV), Cl (2.63 keV) and Cu (8.08 keV).

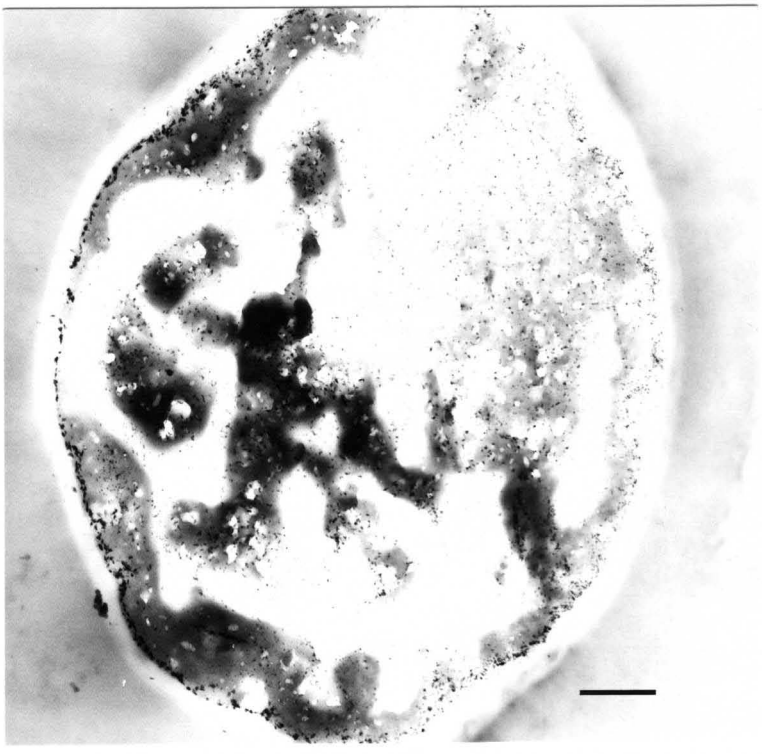

Figure 6.14: A TEM micrograph of a cross section of a *S. cerevisiae* cell after exposure to a lead solution. The lead can be observed as speckled electron areas throughout the cell. Scale bar = 0.5μm.

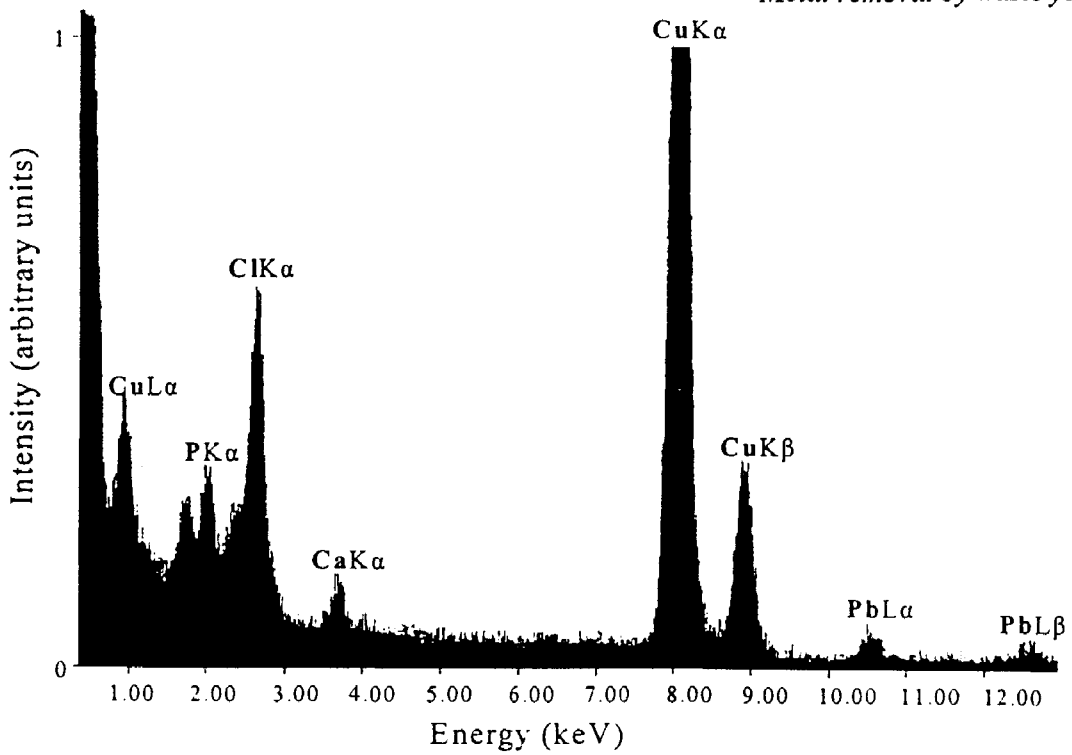

Figure 6.15: Spectrum obtained from the cytoplasm of a cell of *Saccharomyces cerevisiae* exposed to 200μmoles/ℓ of lead. Major identifiable peaks are P (2.01 keV), Cl (2.63 keV), Ca (3.75 keV) and Pb (11.58 and 12.67 keV). The Cu peaks are due to the supporting copper grids.

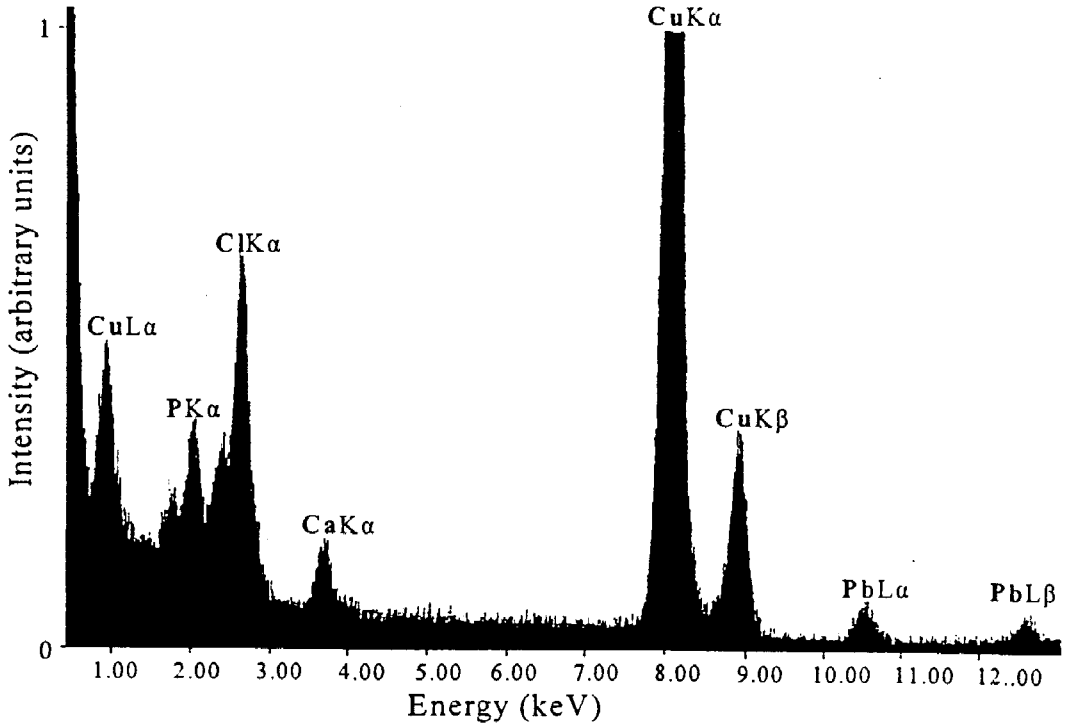

Figure 6.16: Spectrum obtained from the margin (wall) of a cell of *Saccharomyces cerevisiae* exposed to 200μmoles/ℓ of lead. Major identifiable peaks are P (2.01 keV), Cl (2.63 keV), Ca (3.75 keV) and Pb (11.58 and 12.67 keV). The Cu peaks are due to the supporting copper grids.

Cells of *S. cerevisiae* exposed to iron also had better defined cytoplasmic inclusions when compared to the control cells. A micrograph of a cell exposed to iron is presented in Figure 6.17. Relatively small iron peaks were detected in the cell cytoplasm and cell wall by EDXA. These spectra are presented in Figure 6.18 and 6.19 respectively. Small zinc peaks in both the cytoplasm and cell walls were observed from X-ray analysis of zinc exposed cells (spectra are not shown).

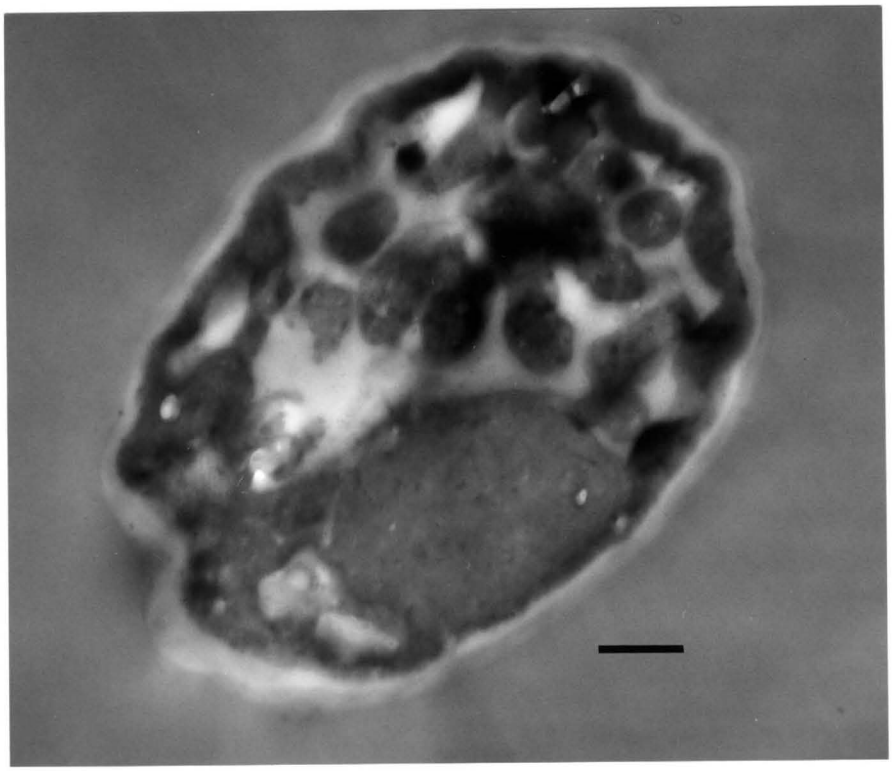

Figure 6.17: A TEM micrograph of a cross section of a *S. cerevisiae* cell after exposure to an iron solution. Scale bar = 0.5μm.

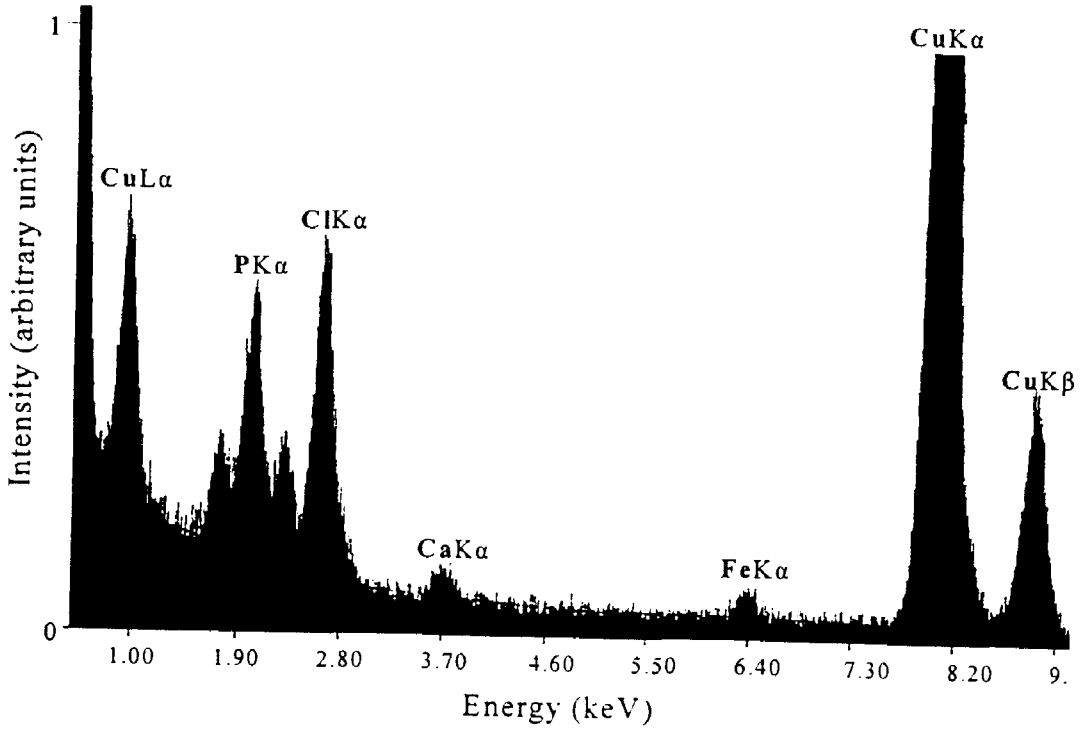

Figure 6.18: Spectrum obtained from the cytoplasm of a cell of *Saccharomyces cerevisiae* exposed to 200μmoles/ℓ of iron. Major identifiable peaks are P (2.01 keV), Cl (2.63 keV), Ca (3.75 keV) and Fe (6.43 keV). The Cu peaks are due to the copper grids.

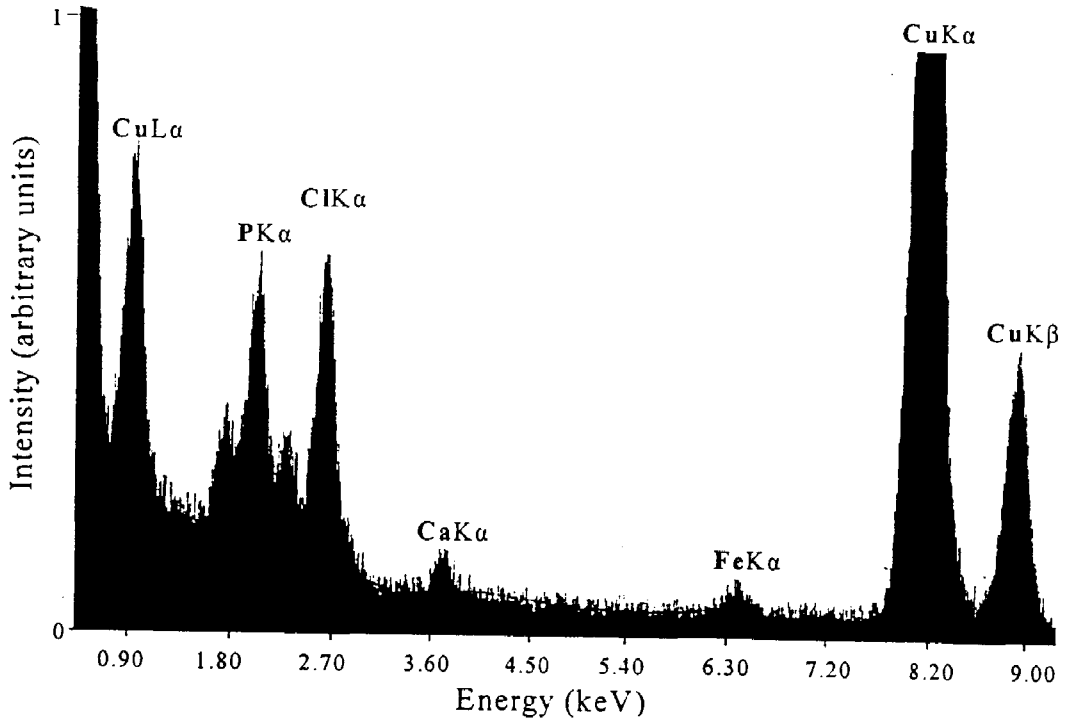

Figure 6.19: Spectrum obtained from the margin (wall) of a cell of *Saccharomyces cerevisiae* exposed to 200μmoles/ℓ of iron. Major identifiable peaks are P (2.01 keV), Cl (2.63 keV), Ca (3.75 keV) and Fe (6.43 keV). The Cu peaks are due to the supporting copper grids.

6.4 DISCUSSION

6.4.1 Yeast physiology and conditioning

Waste *S. cerevisiae* was obtained as a slurry from a local commercial brewery post fermentation. An estimate of waste yeast generated by the brewery was calculated at approximately 30 000 kg per week which could potentially provide sufficient biomass for a bioremediation plant at a low cost.

The waste yeast was pitched and reused in up to 6 different fermentations before being discarded and was generally in a poor physiological condition. Differences in the condition of the waste yeast could occur due to different fermentation conditions and could potentially affect metal removal. Physiological assays, commonly used at the breweries, were used for rapid assessment of yeast condition, which included; pH measurement, a viability test using methylene blue staining, and a protease assay. The different batches of yeast collected from the breweries were at pH $\geq$ 5.5. A yeast slurry post fermentation of pH $\geq$ 5.0 indicates cell stress. The viability of the cells varied considerably between the different batches. Methylene blue staining indicated cell viabilities ranged from 50 to 85 %. Protease assays were performed in conjunction with pH and methylene blue stain measurements for a more conclusive assessment of the physiological condition of the yeast. The protease assay measures the excretion products in the supernatant post centrifugation of the yeast slurry due to the autolysis of non-viable cells. Absorbance differences ranged from 0.04 to 0.02 and corresponded to the cell viabilities of 50 and 85 %, respectively obtained earlier. Accepted brewing standards require an absorbance difference of $\leq$ 0.01 for pitching yeast. Discrepancies may arise in viability assays due to different criteria of assessing cell death. A particular cell may for example be incapable of division but may still be capable of active metabolism, leading to uptake of substrates and release of metabolites to the media (Hough *et al*, 1971). Thus viability assays should be referenced and the results interpreted according to the criteria of the assay. The three rapid assays performed in this investigation consistently indicated a poor physiological yeast condition.

Variations in metal bioremediation performance were observed between different batches of yeast. However, these variations did not appear to be related to the physiological status of the yeast. Metal removal capacities of different batches of yeast collected over an 18 month period varied randomly and, as an example, a batch of yeast which had the lowest recorded cell viability count of 50 %, had an average copper B_{max} of 25.4 μmol/g compared to 25.0 μmol/g and 26.3 μmol/g for yeast with cell viability counts of $\geq$ 80 %. Singleton and Simmons (1996) reported a similar result and found that the metabolic state of brewer's yeast had no effect on silver biosorption. Volesky *et al* (1993) reported a substantial difference in the uptake of cadmium depending on physiological status with fresh exponential growth phase cells accumulating greater quantities of the metal. Mochaba *et al* (1996b) observed that trace metal ions of iron, manganese and copper were absorbed and distributed intracellularly by yeast according to physiological status and growth stage of the yeast.

Due to the variances in the biomass metal uptake capacities between batches collected at different intervals as shown in table 6.2, a wash protocol was established to obtain consistent yeast performance. Mochaba *et al* (1996) reported the iron and copper in wort differed substantially among different brews in the same brewhouse. The accumulation of metals during the fermentation process would potentially impact negatively on the yeasts performance as a biosorbent. The acid conditioning was similar to the desorption protocol used for metal saturated, immobilized yeast (Section 3.2.3) and improved metal removal up to 100 %. However, only trace amounts of copper (0.24 μmol/g) were detected from the acid wash. A compound was probably removed or altered by conditioning but was undetected and requires further investigation. It may be of importance to consider the fermentation methods of the brewery which may be causing the variation in biomass performance. Unfortunately inquiries in this regard in this study did not yield the information required. The practical implications of an initial conditioning step, however, would be negligible as it would require the same infrastructure and chemicals for metal desorption, which would already be included in the process. Simmons *et al* (1995) found that individual batches of waste biomass obtained from a Dublin

brewery, and produced from different fermentation runs, had consistent metal uptake capacities.

The waste brewer's yeast cells were in a non-proliferating stationary phase. Entry into a starvation-induced stationary phase allows long term maintenance of viability with the cells undergoing metabolic and morphological changes. Of significance to metal adsorption is the thickening of the cell wall of stationary phase cells which is observed in the control cell micrographs. The cell wall mannoproteins which surround the glucan polysaccharide matrix have altered N-glycosylation and contain increased numbers of disulphide bridges. The altered mannoprotein confers resistance to enzymatic activity, possibly a selective survival mechanism to proteases released from autolysed cells (Werner-Washburne *et al*, 1993).

6.4.2 Biomass concentration

A yeast concentration of 10 mg/mℓ was used for batch reactor investigations. A concentration above this value only gave negligible improvements in the percentage metal removal, however metal accumulation per gram of biomass was substantially decreased (Refer to Table 6.1). The biomass concentration which yielded a satisfactory residual metal concentration was selected. Singleton and Simmons (1996) found increasing the yeast concentration from 1 mg/mℓ to 8 mg/mℓ (dry wt) decreased silver accumulation from 224.7 μmol/g to 89.5 μmol/g. However, total silver removal from solution would have been approximately 3 times higher at a biomass concentration of 8 mg/mℓ According to Singh *et al* (1992), higher cell populations result in reduced metal uptake owing to the lower metal ion concentrations per unit mass in dense cell mixtures.

6.4.3 Metal removal over time

The rates of metal uptake by the brewer's *S. cerevisiae* were rapid. Metal association with the cells was investigated to establish the time required for binding equilibrium to be reached for the kinetic fixed time assays which followed. Centrifugation of the cells for separation from the metal solution increased the exposure time and, therefore, incubation

times of 15 min and longer were used. Copper removal reached 14.7 μmol/g after 15 min and increased slightly to 15.5 μmol/g after 360 min. Lead removal was 13.1 μmol/g and had a similar profile to copper. Zinc removal by the yeast was low reaching 6.5 μmol/g after 120 min. Of the metals investigated only iron removal increased over time from an initial 6.9 μmol/g after 15 min and 9.5 μmol/g after 9.5 μmol/g. Singleton and Simmons (1996) reported no marked increase in silver uptake by waste brewer's *S. cerevisiae* after 5 min. Volesky and May-Phillips (1995) reported uranium uptake by *S. cerevisiae* to be rapid, with 60 % of total removal occurring in the first 15 min. Cadmium uptake by *S. cerevisiae* was found to be 73 % complete within 3 min of contact (Volesky *et al*, 1993). Similar rapid accumulation of cations has been reported for other microorganisms (Fourest and Roux, 1992; Al-Asheh and Duvnjak, 1995).

6.4.4 pH and metal removal

Metal uptake by the brewer's *S. cerevisiae* was dependent on initial metal solution pH. Investigations on lead and iron above pH 5.5 and pH 7.0 for copper and zinc were not possible due to metal precipitation which could not be differentiated from bioaccumulation. Similar removal profiles between a pH range of 2.0 to 6.0 were obtained for each of the four metals. At a pH above 4.0, metal removal was unaffected and remained similar to the removal efficiencies at the natural solution pH. At an initial pH 3.0 metal uptake decreased. A pH of 5.0 was used in subsequent biosorption studies as it required limited adjustment from natural pH and precipitation was not a contributing factor.

The importance of pH in the biosorption process has been reported by several researchers (Fourest *et al*, 1994; Golab *et al*, 1991; Ramelow *et al*, 1992; Nui *et al*, 1993). At higher solution pH a negative surface potential is created by dissociation of acidic groups causing an effective increase in cation concentration at the surface membrane (Jones and Gadd, 1990). Jones and Gadd (1990) reported a competitive inhibition between cations and H^+ at pH below 4, a non-competitive inhibition at pH 4 to 6 and little or no effect at pH 6 to 8.

6.4.5 Biosorption isotherms

The uptake capacity of metal by the brewer's yeast was investigated in batch reactors using a fixed time assay of 30 min over a concentration range of 100 to 500 μmol/ℓ. The binding isotherms represent the equilibrium of metals in solution and metals accumulated to the biosorbent. Linear transformation of the equilibrium isotherms was used to determine the maximum binding (B_{max}) of metals and is a useful parameter for comparison of removal potential by the biosorbent between the various metals. The maximum removal capacity was highest for copper at 25.4 μmol/g, followed by lead at 19.4 μmol/g, iron at 15.6 μmol/g and zinc at 12.5 μmol/g.

The relatively good uptake capacities from low metal concentration solutions by the brewer's yeast suggest it could be utilized in the treatment of high volume relatively low concentration effluents. Volesky *et al* (1993) reported a slightly higher uptake of cadmium by baker's yeast in comparison to brewer's yeast. Volesky and May-Phillips (1995) reported a high removal capacity of uranyl ions by viable and non-viable brewer's *S. cerevisiae*, with baker's yeast removing twice the amount of zinc as non-viable brewer's yeast.

6.4.6 Transmission electron microscopy and EDXA

Bioaccumulation of metal by the *S. cerevisiae* cells was confirmed by electron microscopic examinations and energy dispersive X-ray analysis. Conditioned control cells had large clear cell wall regions and the cytoplasms of these controls were relatively undefined. The cell walls of metal exposed cells were not as prominent as the controls. The clarity of the micrographs was diminished due to the relative thickness (320 nm) of the sections and the lack of staining. This is required in the preparation process to improve sensitivity and prevent interference during X-ray analysis.

Copper exposed *S. cerevisiae* cells had electron dense regions on the cell wall and within the cytoplasm. Well defined copper peaks were obtained from the X-ray analysis of the cytoplasm and the cell wall region. Mochaba *et al* (1996a) found copper was mostly

concentrated in the cytosol of brewer's yeast. It was not clear from the micrographs or the X-ray analysis if the copper was evenly distributed throughout the cells or localized in defined areas. Jones and Gadd (1990) observed that cations such as copper can bind to intracellular ligands causing toxicity. Intracellular uptake of copper has also been associated with cell vacuoles (Yazgan and Özcengiz, 1994).

Micrographs of the lead exposed cells showed lead accumulated throughout the cell which could be observed as speckled electron dense areas. Lead deposits appeared more prominent in the cell wall region. These electron dense specks may represent microprecipitation of the lead within the cell structure. Lead accumulation was confirmed by X-ray analysis. Lead was not compartmentalised within the cytoplasm and the peaks obtained from the EDXA spectra from the cell wall region appeared more pronounced. Golab *et al* (1991) observed from an electron microscopic study of lead exposed Streptomyces cells that lead accumulation was primarily to the cell surface structures. Surface binding of lead to *Undaria pinnatifida* cells was also observed and confirmed by EDXA by Kim *et al* (1995).

As with the copper exposed cells, electron micrographs of cells exposed to zinc showed electron dense areas throughout the cell cytoplasm and cell wall. The presence of the metal in these areas of the cells was confirmed by X-ray analysis, although peaks obtained on the X-ray spectra were small. Mochaba *et al* (1996b) found zinc concentrated in the mannoprotein of the cell wall and in the cytoplasm of brewer's yeast post-fermentation. Wavelength dispersive X-ray analysis confirmed the deposition of zinc on the cell surface of *Pseudomonas stutzeri* cells (Bhagat and Srivastava, 1994).

Electron micrographs of cells exposed to iron showed electron dense regions throughout the cell cytoplasm and cell wall region. The presence of iron in the cells was confirmed by X-ray analysis. Mochaba *et al* (1996a) reported that iron was mostly concentrated in the mannoprotein of the cell wall and to a lesser extent in the cytosol of brewer's yeast. Raguzzi *et al* (1988) reported the intracellular distribution of iron was largely associated

with the vacuoles of *S. cerevisiae* cells.

6.5 CONCLUSION

Brewer's *S. cerevisiae* accumulated metals with maximum binding capacities in the order of copper > lead > iron > zinc. The uptake of metals was rapid and pH dependent. While the waste yeast was generally in a poor physiological state, a correlation between cell physiology and metal uptake was not observed in the conditioned biomass. The location of the metals in both the cell cytoplasm and the cell wall was confirmed by EDXA.

The data suggests the waste yeast may be a potential biosorbent for the bioremediation of low concentration, high volume, metal contaminated, waste waters. As waste waters often contain more than one metal, biosorption from dual metal solutions was investigated. The findings are reported in chapter VII.

CHAPTER VII

BIOSORPTION OF METALS FROM MULTI-ION SOLUTIONS BY WASTE *Saccharomyces cerevisiae*

7.1 INTRODUCTION

The research on uptake of metals by microorganisms has largely focused on the removal of single metal ion species from aqueous solutions (Beveridge and Murray, 1976; Lesuisse *et al.*, 1990; Fourest *et al*, 1994). Industrial waste waters, however, seldom contain only one metal ion species. More common are waste waters containing many metal ions and a range of co-contaminants (Galun *et al*, 1984) The presence of a multiplicity of metals leads to interactive effects between the metals and the biosorbent. The interaction of two or more metals may cause changes to the solution chemistry which can affect the physiological and biochemical processes of the biosorbent (Săg and Kutsal, 1996a). The parameters influencing metal removal are considerable and changing a single parameter may result in a decrease in metal removal efficiency.

The removal efficiencies from mixed metal solutions depend on factors such as the number of metals competing for binding sites, metal combinations, metal concentration, mixing times and solution pH. The interactive effects of these solutions can be complex. However, Ting *et al* (1991) simplified the interpretation of data into the following 3 types of responses:

i. synergistic, where the removal of metal from the mixture is greater than the removal of the metals from single ion solutions;

ii. antagonistic, where the removal of metal from the mixture is less than the removal of the metals from single-ion solutions;

iii. non-interactive, where the removal of metal from the mixture is equivalent to the removal of the metals from individual single-ion solutions.

It is postulated that the synergistic response is a result of an increase in the membrane permeability. The antagonistic interaction may be due to a screening effect or a direct competition for binding sites on the cellular surfaces (Ting *et al*, 1991).

Metal uptake is initially associated with cell wall binding followed by transport into the cytoplasm. As metal contact is first with the cell wall, the initial competition would be for the negative functional groups characteristic of yeast cell walls. The metal with the highest affinity for these groups would initially be removed with the highest efficiency. The ability to be taken up into the cytoplasm may require secondary criteria, such as an oxidation or hydrolysis step (Tsezos and Volesky, 1982a). Metal ions binding to the cell wall at a lower concentration may thus be removed at a higher efficiency, if preferentially transported into the cytoplasm.

The objectives of the dual metal investigations were to elucidate the mechanisms by which lead, zinc and iron interfere with the biosorption of copper, and to determine the effect of copper on the biosorption of lead, zinc and iron. The brewer's yeast and copper solutions of known initial concentration were incubated in batch reactors in the presence of increasing concentrations of the second metal (lead, zinc or iron). The reciprocal experiments were then performed using either lead, zinc or iron as the primary metal and copper as the interfering second metal. The biosorption from the binary metal solutions was compared to controls containing only the primary metal.

When more than one metal is present in a sorption system the evaluation, interpretation and representation of the biosorption may become complicated (Figuiera, 1997; Chong and Volesky, 1996). Interpretation and presentation of data was maintained as concise as possible by combining the analysis methods reported by different researchers. This included the using Michaelis-Menten inhibition kinetics, using absolute removal values and the use of metal atomic percentage ratios from X-ray analysis. Michaelis-Menten kinetics were used to determine the affinity and uptake capacity of the brewer's yeast, as used by Gadd and Lawrence (1996) to determine competitive or non-competitive inhibition of

magnesium on the uptake of manganese by *S. cerevisiae.* A second evaluation of the metal uptake by the yeast cells was to determine absolute metal uptake at equimolar initial concentrations from mixed metal solutions and to compare this to equimolar single-ion uptake (Săg and Kutsal, 1996a and b; De Carvalho *et al*, 1995). De Carvalho *et al* (1995) noted that although two metals in solution will mostly interact with each other, and generally cause a decrease in the uptake of individual metals, the total amount of metal sequestered by the biosorbent may increase when exposed to two metals simultaneously. The total uptake was determined by the equation: $q\sum = q^{metal\ 1} + q^{metal\ 2}$, where q = μmol metal uptake per g yeast.

The comparative Michaelis-Menten kinetic and the total uptake data were used to evaluate the extent of synergistic or antagonistic interaction between the metals as proposed by Ting *et al* (1991). Metal deposition sites were determined using transmission electron microscopy and energy dispersive X-ray analysis as used by Tsezos *et al* (1997) for determining aluminum interference of uranium binding. In addition, the ratio of atomic percentages of accumulated metals were used to determine the relative binding to the cell wall and uptake into the cytoplasm.

The combined kinetic and EDXA results were used to interpret the mechanisms of sorption of the different metals to the brewers yeast. The results obtained from the above investigations were compared to a batch reactor bioremediation study of a mine effluent containing the metals copper, lead, zinc and iron.

7.2 MATERIALS and METHODS

7.2.1 Preparation of *S. cerevisiae* for biosorption

As described in section 6.2.1 and 6.2.2.

7.2.2 Binary metal studies

7.2.2.1 Copper and lead

The adsorption characteristics of copper were determined over an initial concentration range of 100, 200, 300, 400 and 500 μmol/ℓ. The lead concentrations in the biosorption mixtures were 0, 100 and 200 μmol/ℓ. The pH of metal mixtures was adjusted to pH 5 with 0.1 M NaOH and 0.1 M HCl. The incubation in batch reactors was as described in section 6.2.3, with an incubation time of 30 min. Metal analysis was as described in section 2.2.6. The presence and influence of the second metal was accounted for by designating apparent values for the dissociation constant and maximum uptake as Kd^{app} and B_{max}^{app} respectively.

To determine the adsorption characteristics of lead, the same experimental procedure was followed as above with the lead range being 100, 200, 300, 400 and 500 μmol/ℓ, and the copper concentration was 0, 50 and 100 μmol/ℓ. The interference of the second metal was concentration dependent and the concentrations were adapted accordingly.

7.2.2.2 Copper and zinc

The adsorption characteristics of copper were determined as described in 7.2.2.1. The zinc concentrations in the biosorption mixtures were 0, 100 and 200 μmol/ℓ. To determine the adsorption characteristics of zinc, the same experimental procedure was followed but the copper concentration was 0, 25 and 50 μmol/ℓ.

7.2.2.3 Copper and iron

The adsorption characteristics of copper were determined as described in 7.2.2.1. The iron concentrations in the biosorption mixtures were 0, 100 and 200 μmol/ℓ. To determine the adsorption characteristics of iron, the same experimental procedure was followed but the copper concentration was 0, 100 and 200 μmol/ℓ.

Appropriate controls were used for all the mixed metal biosorption experiments to

determine any metal adsorption to the glassware and the effect of any mixed metal interactions. The mixed metal solutions were prepared on the day of use and the concentrations checked by atomic absorption analysis prior to biosorption experiments. The controls were used as the initial concentration for uptake calculations, as described in section 6.2.6.

7.2.3 Transmission Electron Microscopy

Cells were incubated in batch reactors containing mixed metal solutions of copper and lead; copper and zinc; and copper and iron. The concentration of each metal was 200 μmol/ℓ. Specimens were prepared as described in 6.2.6.

7.2.4 Energy Dispersive X-ray analysis

As described in 6.2.7. The atomic ratios of the metals in the cells were obtained by using the technique of energy dispersive X-ray microanalysis in a 120 kV Philips EM420 coupled to a EDAX DX-4 energy dispersive X-ray system. The 1996 version of the EDAX DX-4 software was employed. Quantification was done using the standardless thin foil model which includes the absorption correction and with theoretical k factor calculated according to Zaluzec (Williams and Carter, 1996). Only the metallic elements present in the spectrum were used in the quantification and the atomic percentages obtained were then used to calculate the concentration ratios of the various metals present in the yeast cells.

7.2.5 Bioremediation of metals from mine effluent

The mine effluent was as described in 5.2.1 and 5.4.1. The pH of the waste water was adjusted to pH 4 using 1.0 M NaOH. The bioremediation of the metals was investigated at yeast concentrations of 10 mg/mℓ and 20 mg/mℓ. The effluent (9 mℓ) and yeast (1 mℓ) were incubated in batch reactors with shaking for 30 min. Controls contained 9 mℓ effluent and 1 mℓ water.

7.3 RESULTS

7.3.1 Biosorption from binary mixtures of copper and lead

The effects of lead on the removal of copper are presented in Figures 7.1 and 7.2. The B_{max} of copper in a single-ion system was 25.3 μmol/g and the Kd value 152 μmol/ℓ. In the presence of lead at an initial concentration 100 μmol/ℓ, the B_{max}^{app} was 25.0 μmol/g and the Kd^{app} was 207 μmol/ℓ. At 200 μmol/ℓ lead, the B_{max}^{app} was 25.5 μmol/g and the Kd^{app} was 242 μmol/ℓ.

Lead removal was decreased in the presence of copper as shown in Figures 7.3 and 7.4. In a single-ion system, maximum lead removal was 19.2 μmol/g and the Kd 108 μmol/ℓ. At a initial copper concentration of 50 μmol/ℓ, B_{max}^{app} decreased to 14.0 μmol/g and the Kd^{app} value was 87 μmol/ℓ. At 100 μmol/ℓ copper, B_{max}^{app} was 13.5 μmol/g and the Kd^{app} value was 118 μmol/ℓ.

Metal uptake of copper and lead from single-ion, and total metal removal from dual-ion biosorption investigations, are presented in Table 7.1. The metal removal by the brewer's *S. cerevisiae* from single metal solutions, at initial metal concentrations of 200 μmol/ℓ and 400 μmol/ℓ, are presented and compared to metal removal from mixed copper and lead

Table 7.1: The removal of copper and lead from single and mixed metal solutions by brewer's *Saccharomyces cerevisiae*. The initial concentrations of lead and copper are shown in brackets.

Metal	Metal Removal (μmol/g)	
$Cu_{(200\ \mu mol/\ell)}$	13.8 ± 0.54[a]	
$Pb_{(200\ \mu mol/\ell)}$	13.1 ± 0.19[a]	
$Cu_{(400\ \mu mol/\ell)}$	17.4 ± 1.53[a]	Total removal from
$Pb_{(400\ \mu mol/\ell)}$	15.3 ± 0.34[a]	mixed metal solutions
$Cu_{(200\ \mu mol/\ell)}Pb_{(200\ \mu mol/\ell)}$	11.5 ± 0.40[a]	(μmol/g)
$Pb_{(200\ \mu mol/\ell)}\ Cu_{(200\ \mu mol/\ell)}$	6.6 ± 0.16[a]	18.1

a - Mean ± SD of 3 determinations

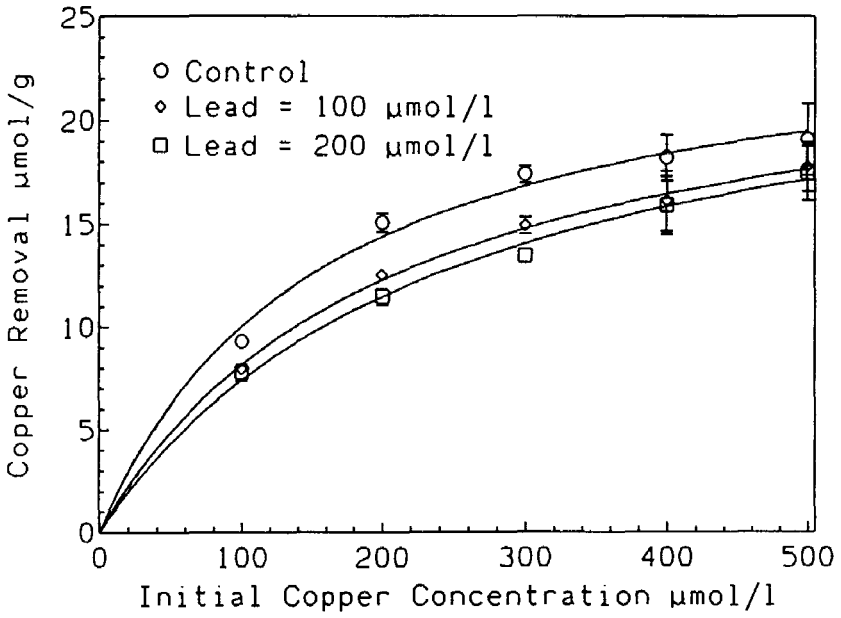

Figure 7.1: Sorption isotherms of initial copper concentrations and the effect on the copper removal with the addition of lead by brewer's *Saccharomyces cerevisiae* (n=6, ± SD). The incubation time was 30 min and the yeast concentration was 10 mg/mℓ. Lead concentrations were 0, 100 and 200 μmol/ℓ.

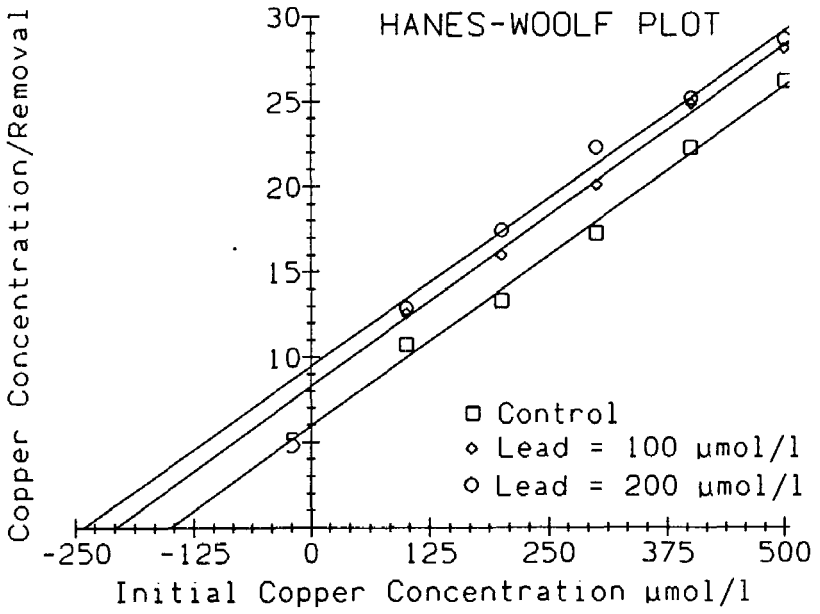

Figure 7.2: Hanes-Woolf plots showing the effect of lead on the removal of copper. Maximum removal (B_{max}) of copper with no lead present was 25.3 μmol/g, in the presence of 100 μmol/ℓ lead the B_{max} was 25.0 μmol/g and 25.5 μmol/g in the presence of 200 μmol/ℓ lead. Kd values increased with increasing lead concentrations.

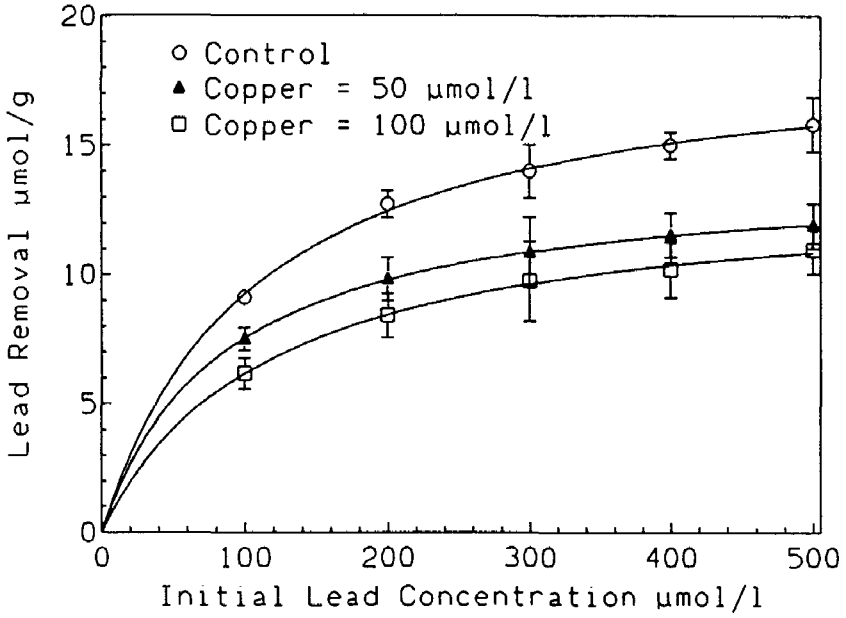

Figure 7.3: Sorption isotherms of initial lead concentrations and the effect on the lead removal in the presence of copper by brewer's *Saccharomyces cerevisiae* (n=6, ± SD). The incubation time was 30 min and the yeast concentration 10 mg/mℓ. Copper concentrations were 0, 50 and 100 µmol/ℓ.

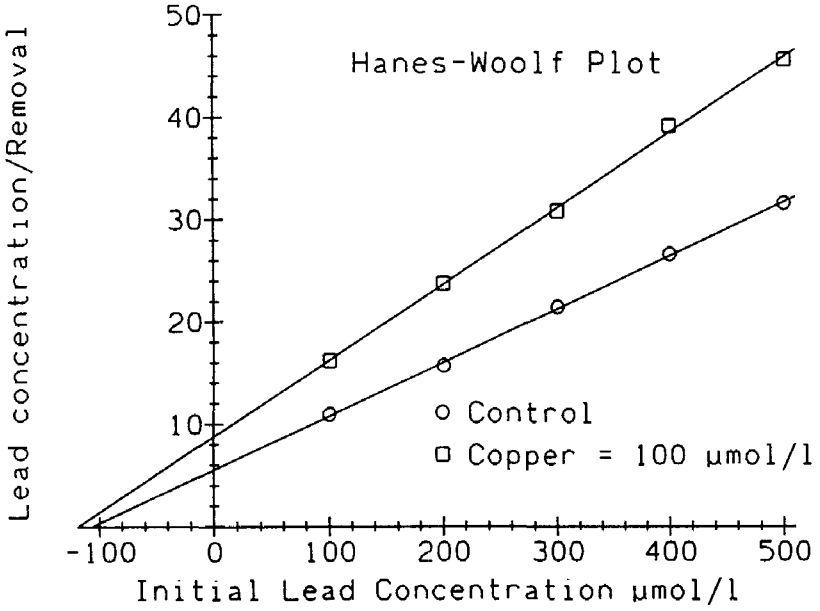

Figure 7.4: Hanes-Woolf plots showing the effect of copper at a on the removal of lead. The B_{max} and Kd of lead removal with no copper present was 19.2 µmol/g and 108 µmol/ℓ respectively. B_{max}^{app} and Kd^{app} were 13.5 µmol/g and 118 µmol/ℓ respectively in the presence of 100 µmol/ℓ copper.

solutions of an initial concentration of 200 μmol/ℓ per metal. The average total removal from the mixed solution was 18.1 μmol/g.

A transmission electron micrograph of a cell taken from a batch reactor containing copper and lead of initial concentrations of 200 μmol/ℓ is shown in Figure 7.5. Electron dense regions occur throughout the cytoplasm and the wall cell. Fine electron dense specks are also seen throughout the cell which were common to the cells exposed to lead. The presence of both copper and lead was confirmed by EDXA. The copper to lead concentration ratios (obtained from the copper and lead atomic percentages) in the cell cytoplasm were mostly found to be higher for copper. The concentration ratios of copper to lead obtained from the analysis of different cells ranged from 3:1, 2:1 to 3:4. Analysis of the cell wall region of different cells consistently gave a higher copper ratio, ranging from 3:1 to 6:5. EDXA spectra from the copper and lead investigation are shown in Figure 7.6 and 7.7.

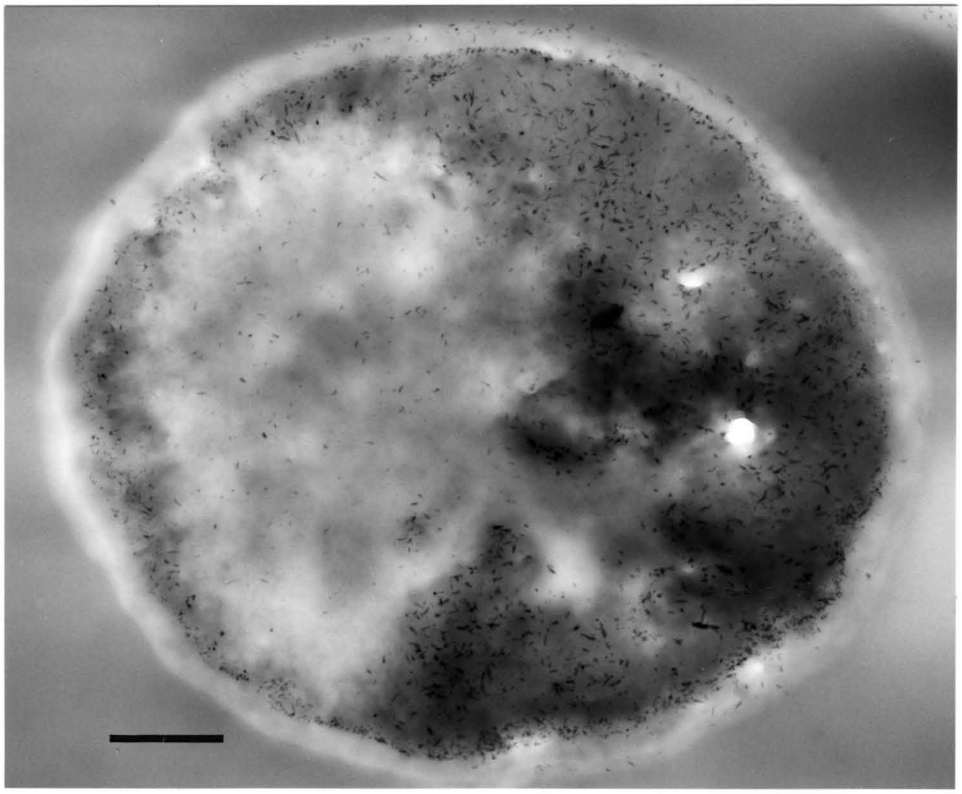

Figure 7.5: A TEM micrograph of a cross section of a *S. cerevisiae* cell after exposure to a mixed solution of copper and lead. The presence of metals in the electron dense areas were confirmed using EDXA. Scale bar = 0.5 μm.

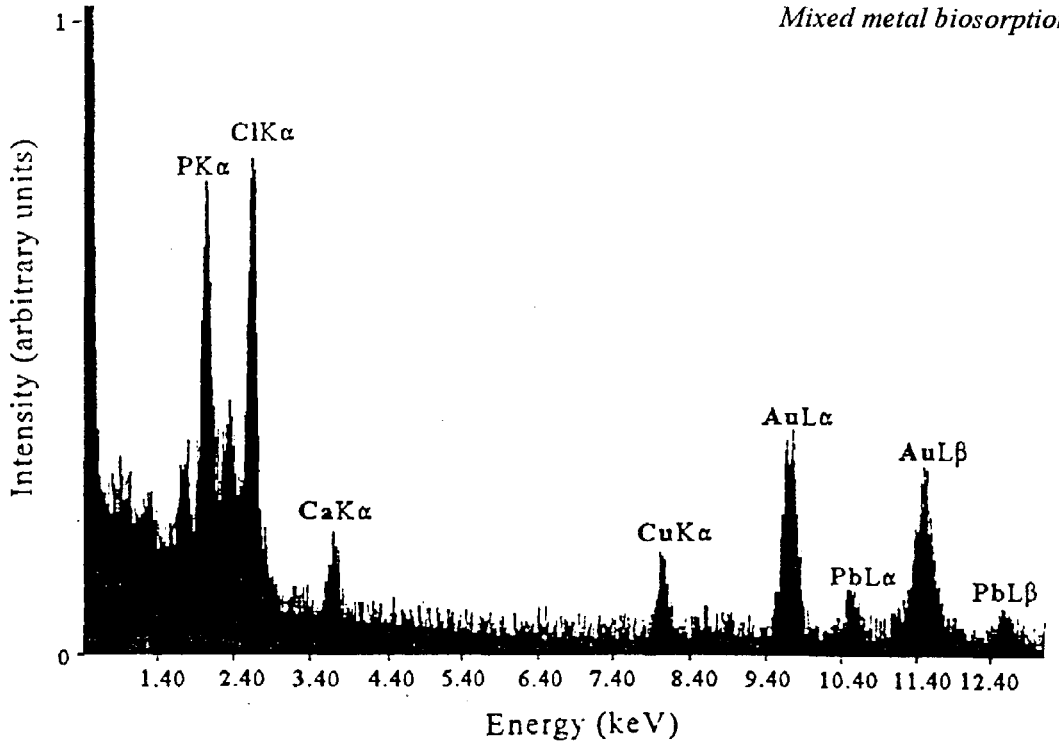

Figure 7.6: EDXA spectrum obtained from the cytoplasm of a cell of *Saccharomyces cerevisiae* exposed to 200μmoles/ℓ of copper and lead. Major identifiable peaks are P (2.01 keV), Cl (2.63 keV), Ca (3.75 keV), Cu (8.08 keV) and Pb (11.58 and 12.67 keV). The Au peaks are due to the supporting gold grids.

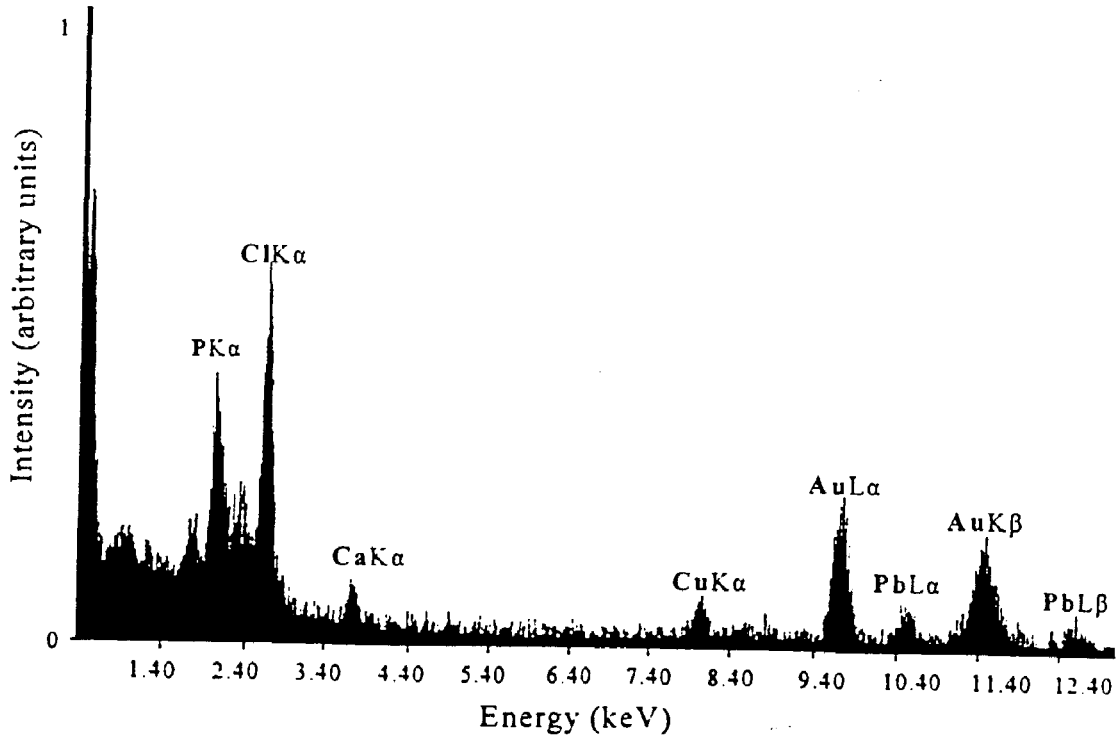

Figure 7.7: EDXA spectrum obtained from the margin (wall) of a cell of *Saccharomyces cerevisiae* exposed to 200 μmoles/ℓ of copper and lead. Major identifiable peaks are P (2.01 keV), Cl (2.63 keV), Ca (3.75 keV), Cu (8.08 keV) and Pb (11.58 and 12.67 keV). The Au peaks are due to the supporting gold grids.

7.3.2 Biosorption from binary mixtures of copper and zinc

The effects of zinc on the removal of copper are presented in Figures 7.8 and 7.9. The zinc appeared to have a slight synergistic effect on copper removal. The B_{max} of copper in a single-ion system was 30.8 μmol/g and the Kd value 211 μmol/ℓ. In the presence of zinc of initial concentration 100 μmol/ℓ, the copper removal B_{max}^{app} increased to 32.3 μmol/g and the Kd^{app} was 228 μmol/ℓ. At 200 μmol/ℓ zinc, copper B_{max}^{app} was 34.5 μmol/g and Kd^{app} was 264 μmol/ℓ.

Zinc removal was decreased in the presence of copper as shown in Figures 7.10 and 7.11. The B_{max} of zinc in a single-ion system was 13.1 μmol/g and the Kd value 111 μmol/ℓ. At a initial copper concentration of 25 μmol/ℓ, the zinc removal B_{max}^{app} decreased to 11.9 μmol/g and the Kd^{app} was 150 μmol/ℓ. At 50 μmol/ℓ copper, the B_{max}^{app} was 10.6 μmol/g and Kd^{app} was 170 μmol/ℓ.

Metal uptake of copper and zinc from single-ion, and total metal removal from dual-ion biosorption investigations, are presented in table 7.2. The average total metal removal from the mixed copper and zinc solutions of an initial concentration of 200 μmol/ℓ was 21.1 μmol/g.

Table 7.2: The removal of copper and zinc from single and mixed metal solutions by brewer's *Saccharomyces cerevisiae*. The initial concentrations of zinc and copper are shown in brackets.

Metals	Metal removal (μmol/g)	
$Cu_{(200\ \mu mol/\ell)}$	15.9 ± 0.08^a	
$Zn_{(200\ \mu mol/\ell)}$	8.3 ± 0.32^a	
$Cu_{(400\ \mu mol/\ell)}$	20.3 ± 0.42^a	Total removal from
$Zn_{(400\ \mu mol/\ell)}$	10.9 ± 1.06^a	mixed metal solutions
$Cu_{(200\ \mu mol/\ell)}$ (+$Zn_{(200\ \mu mol/\ell)}$)	16.0 ± 0.40^a	μmol/g
$Zn_{(200\ \mu mol/\ell)}$ (+ $Cu_{(200\ \mu mol/\ell)}$)	5.1 ± 0.75^a	21.1

a - Mean ± SD of 3 determinations

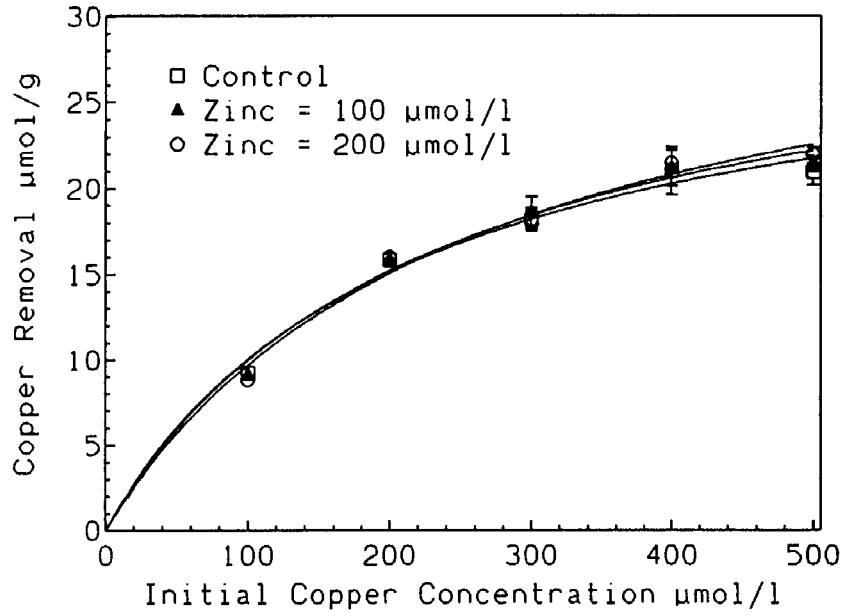

Figure 7.8: Sorption isotherms of initial copper concentrations and the effect on the copper removal in the presence of zinc by brewer's *Saccharomyces cerevisiaie* (n=6, ± SD). The incubation time was 30 min and the yeast concentration 10 mg/mℓ. The zinc concentrations were 0, 100 and 200 μmol/ℓ.

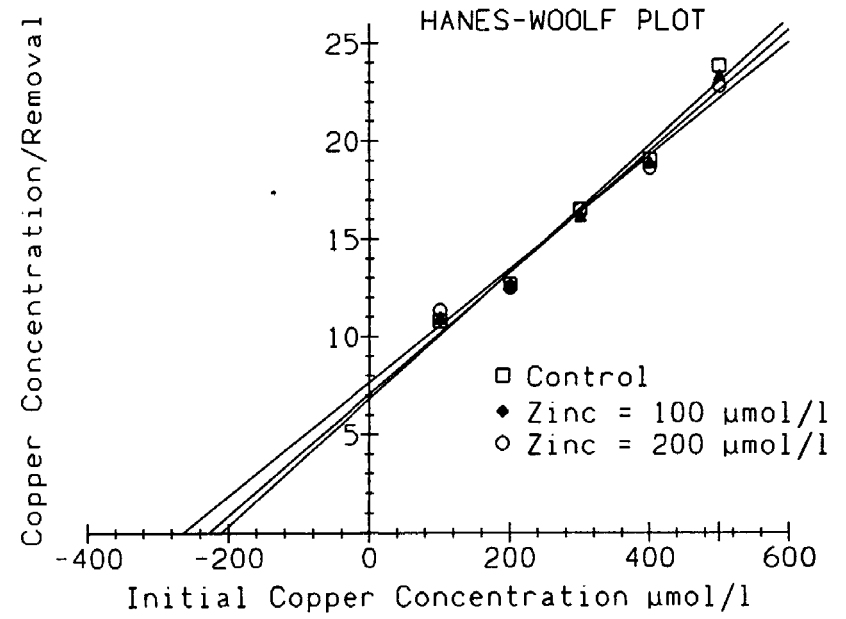

Figure 7.9: Hanes-Woolf plots showing the effect of zinc on the removal of copper. The B_{max} of copper removal with no zinc present was 30.8 μmol/g, in the presence of 100 μmol/ℓ zinc the B_{max}^{app} was 32.3 μmol/g and 34.5 μmol/g in the presence of 200 μmol/ℓ zinc. Kd values increased with increasing zinc concentrations.

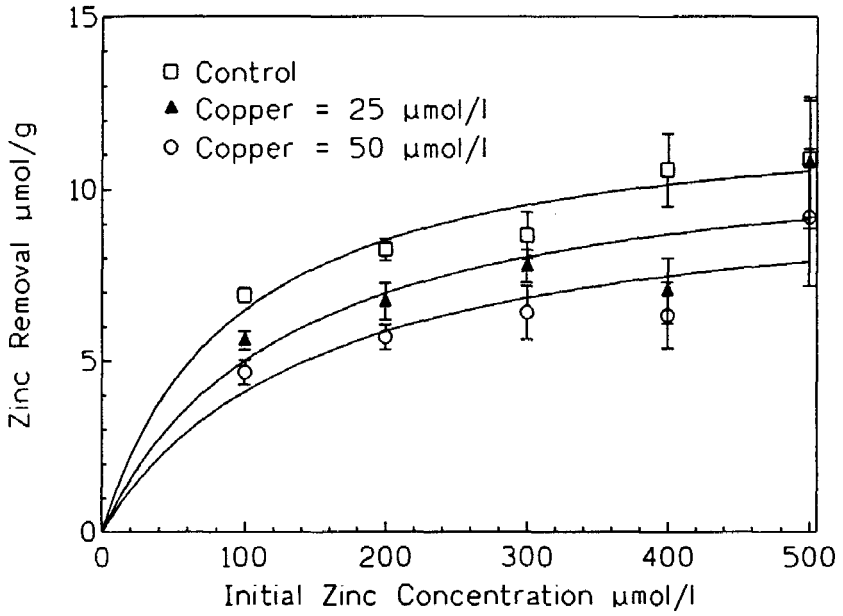

Figure 7.10: Sorption isotherms of initial zinc concentrations and the effect on the zinc removal in the presence of copper by brewer's *Saccharomyces cerevisiae* (n=6, ± SD). The incubation time was 30 min and the yeast concentration was 10 mg/mℓ. The copper concentrations were 0, 25 and 50 μmol/ℓ.

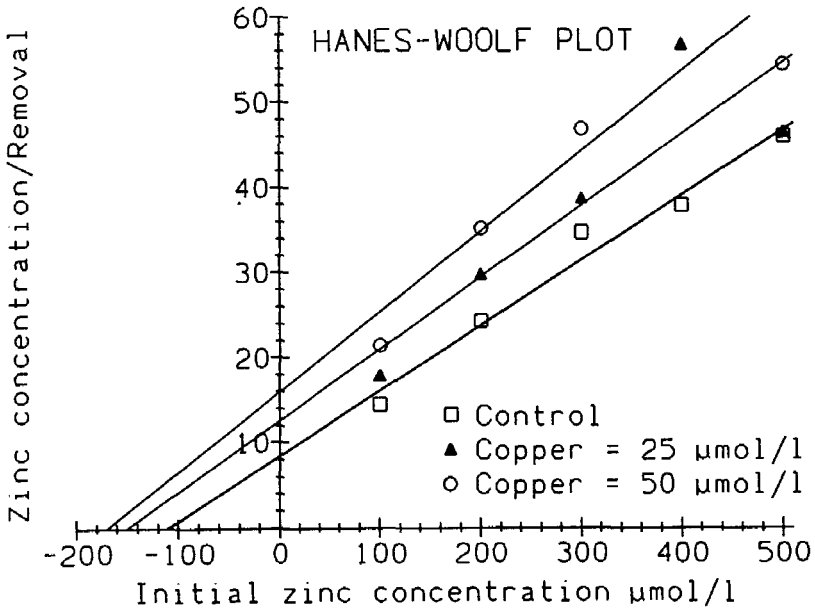

Figure 7.11: Hanes-Woolf plots showing the effect of copper on the removal of zinc. The B_{max} of zinc with no copper present was 13.1 μmol/g, in the presence of 25 μmol/ℓ copper the B_{max}^{app} was 11.9 μmol/g and 10.6 μmol/g in the presence of 50 μmol/ℓ copper. Kd values increased with increasing copper concentrations.

A transmission electron micrograph of a cell taken from a batch reactor containing copper and zinc of initial concentrations of 200 μmol/ℓ is shown in Figure 7.12. The presence of copper and zinc were confirmed by EDXA. The copper to zinc concentration ratios were found to be higher for copper in both the cell cytoplasm and the cell wall. The concentration ratio of copper to zinc of the cell cytoplasm was between 3:1 and 4:1. The ratio of copper to zinc in the cell wall region was 2:1.

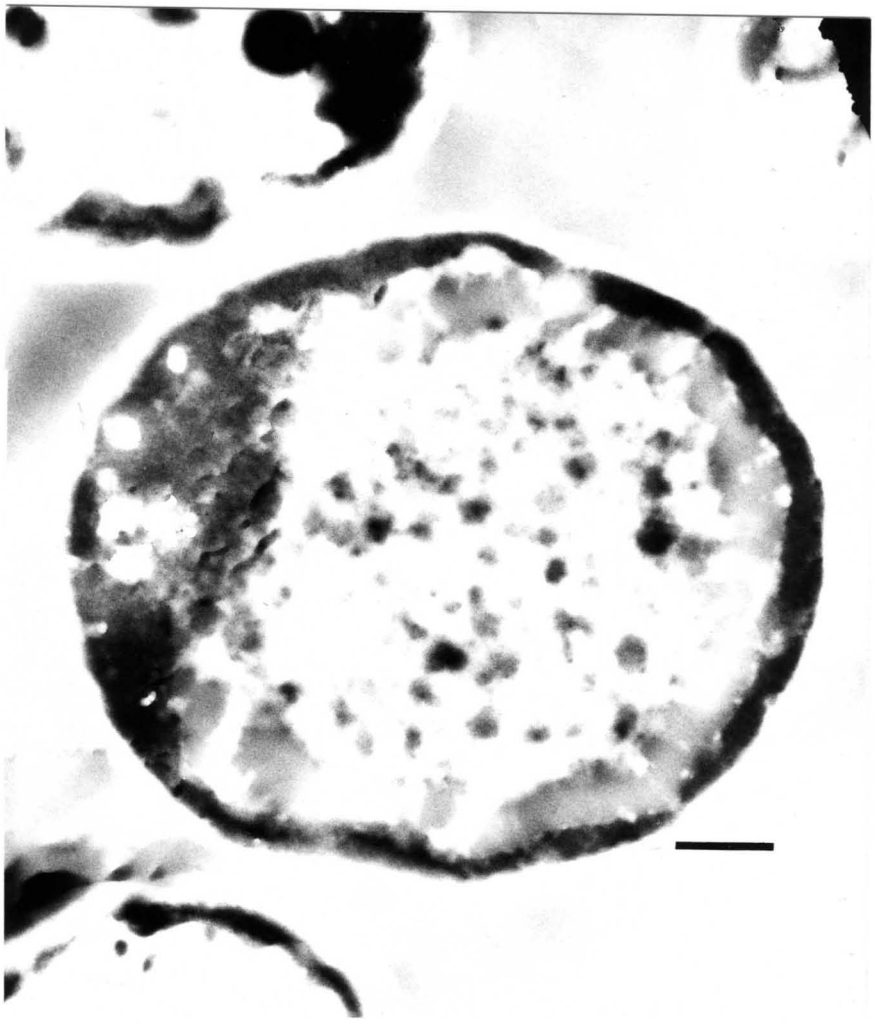

Figure 7.12: A TEM micrograph of a cross section of a *S. cerevisiae* cell after exposure to a mixed solution of copper and zinc. Confirmation of the metals and their relative ratios was determined by EDXA. Scale bar = 0.5 μm.

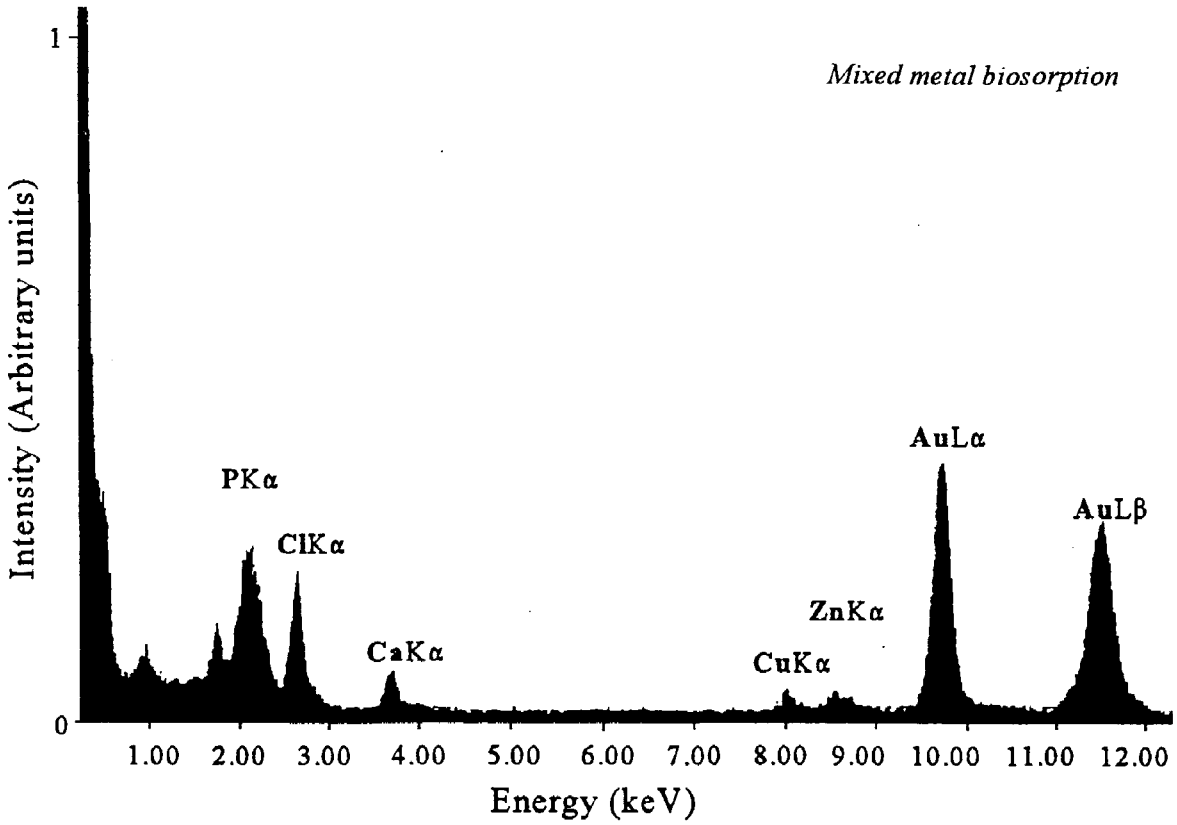

Figure 7.13: EDXA spectrum obtained from the cytoplasm of a cell of *Saccharomyces cerevisiae* exposed to 200μmoles/ℓ of copper and zinc. Major identifiable peaks are P (2.01 keV), Cl (2.63 keV), Ca (3.75 keV), Cu (8.08 keV) and Zn (8.68 keV). The Au peaks are due to the supporting gold grids.

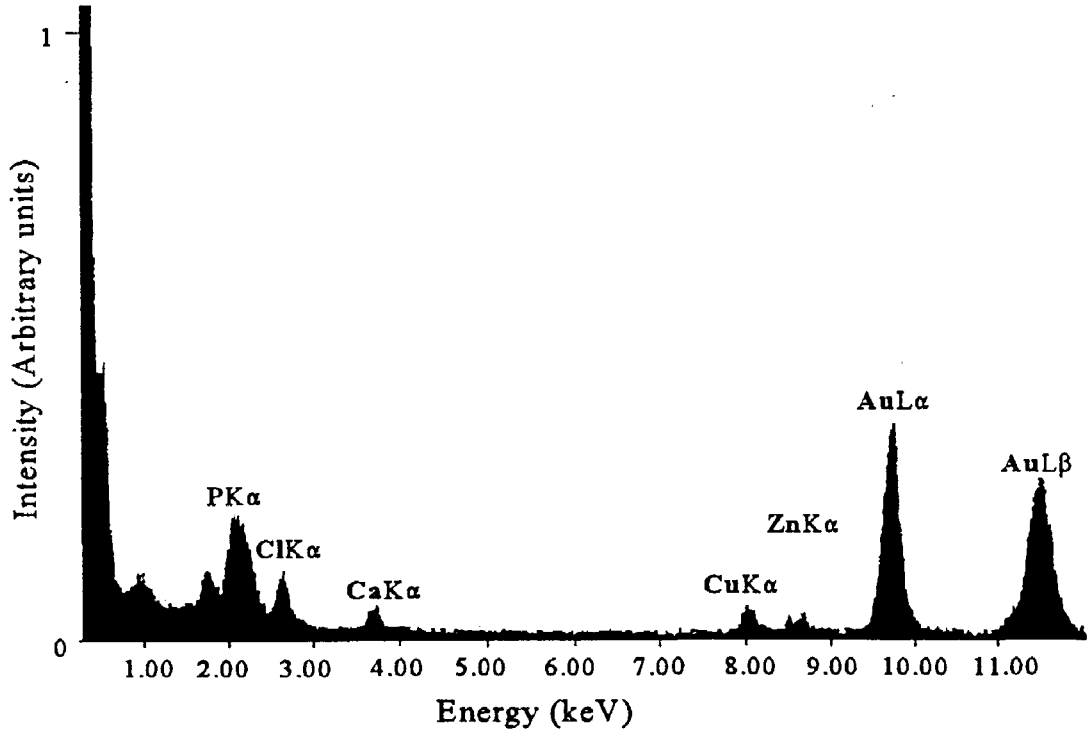

Figure 7.14: EDXA spectrum obtained from the margin (wall) of a cell of *Saccharomyces cerevisiae* exposed to 200μmoles/ℓ of copper and zinc. Major identifiable peaks are P (2.01 keV), Cl (2.63 keV), Ca (3.75 keV), Cu (8.08 keV) and Zn (8.58 keV). The Au peaks are due to the supporting gold grids.

7.3.3 Biosorption from binary mixtures of copper and iron

The effects of iron on the removal of copper are presented in Figures 7.15 and 7.16. Copper uptake was decreased in the presence of iron. The B_{max} of copper in a single-ion system was 25.4 μmol/g and the Kd value 215 μmol/ℓ. At 100 μmol/ℓ iron, the B_{max}^{app} was 21.0 μmol/g and the Kd^{app} was 165 μmol/ℓ. In the presence of 200 μmol/ℓ iron, the B_{max}^{app} was 20.1 μmol/g and the Kd^{app} was 136 μmol/ℓ.

Iron removal was not decreased in the presence of copper as shown in Figures 7.17 and 7.18. In a single ion system, iron removal was 15.5 μmol/g and the Kd 174 μmol/ℓ. At 100 μmol/ℓ copper, the B_{max}^{app} was 15.7 μmol/g and the Kd^{app} was 188 μmol/ℓ. In the presence of 200 μmol/ℓ copper, the B_{max}^{app} was further increased to 16.0 μmol/g and the Kd^{app} was 182 μmol/ℓ.

Metal uptake of copper and iron from single-ion, and total metal removal from dual-ion biosorption investigations, are presented in Table 7.3. The average total removal from the mixed iron and copper solutions of an initial concentration of 200 μmol/ℓ per metal was 21.7 μmol/g.

Table 7.3: The removal of copper and iron from single and mixed metal solutions by brewer's *Saccharomyces cerevisiae*. The initial concentrations of the iron and copper are shown in brackets.

Metals	Metal removal (μmol/g)	
$Cu_{(200\ \mu mol/\ell)}$	13.6 ± 0.42[a]	
$Fe_{(200\ \mu mol/\ell)}$	8.6 ± 0.58[a]	
$Cu_{(400\ \mu mol/\ell)}$	17.6 ± 0.62[a]	Total removal from
$Fe_{(400\ \mu mol/\ell)}$	11.3 ± 0.51[a]	mixed metal solutions
$Cu_{(200\ \mu mol/\ell)}$ (+$Fe_{(200\ \mu mol/\ell)}$)	13.3 ± 0.53[a]	(μmol/g)
$Fe_{(200\ \mu mol/\ell)}$ (+$Cu_{(200\ \mu mol/\ell)}$)	8.4 ± 0.82[a]	21.7

a - Mean ± SD of 3 determinations

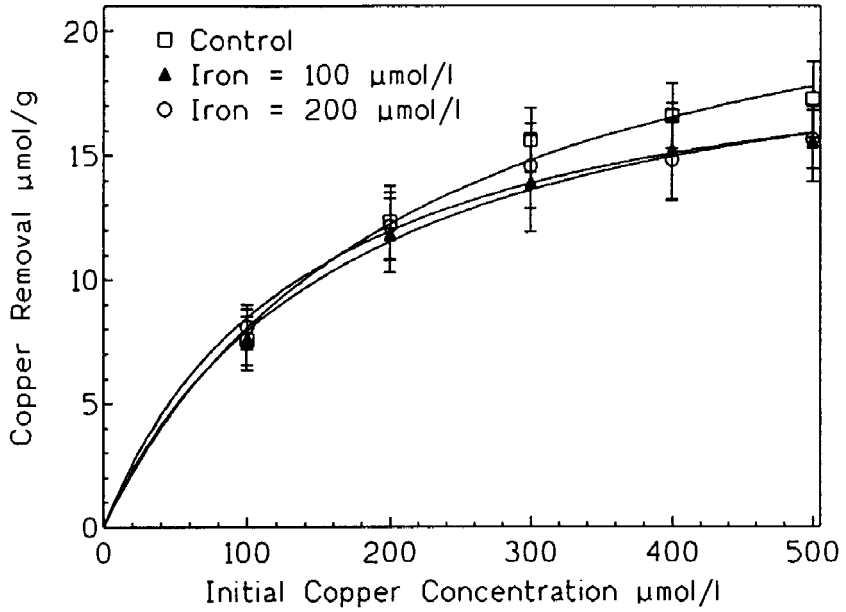

Figure 7.15: Sorption isotherms of initial copper concentrations and the effect on copper removal in the presence of iron by brewer's *Saccharomyces cerevisiae* (n=6, ± SD). The incubation time was 30 min and the yeast concentration 10 mg/mℓ. The iron concentrations were 0, 100 and 200 μmol/ℓ.

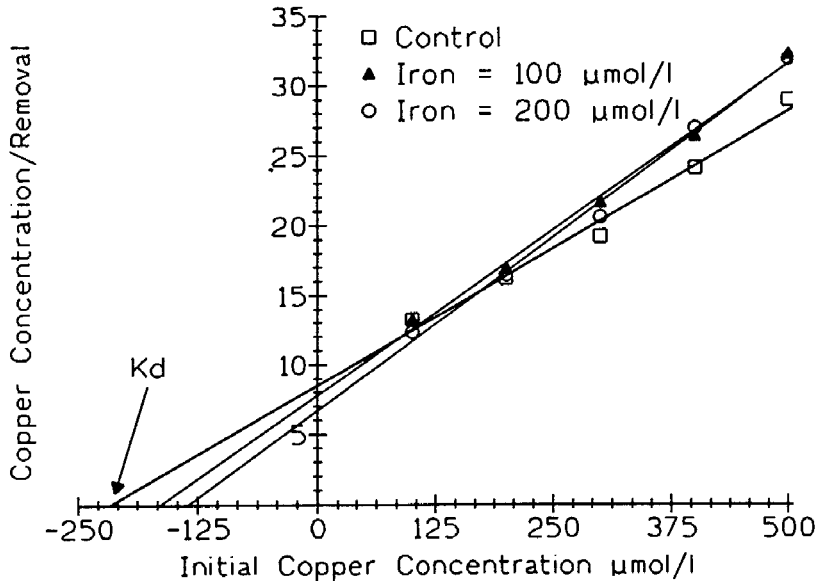

Figure 7.16: Hanes-Woolf plots showing the effect of iron on the removal of copper. The B_{max} of copper with no iron present was 25.4 μmol/g, in the presence of 100 μmol/ℓ iron the B_{max}^{app} was 21.0 μmol/g and 20.1 μmol/g in the presence of 200 μmol/ℓ iron. Kd values decreased with increasing iron concentrations.

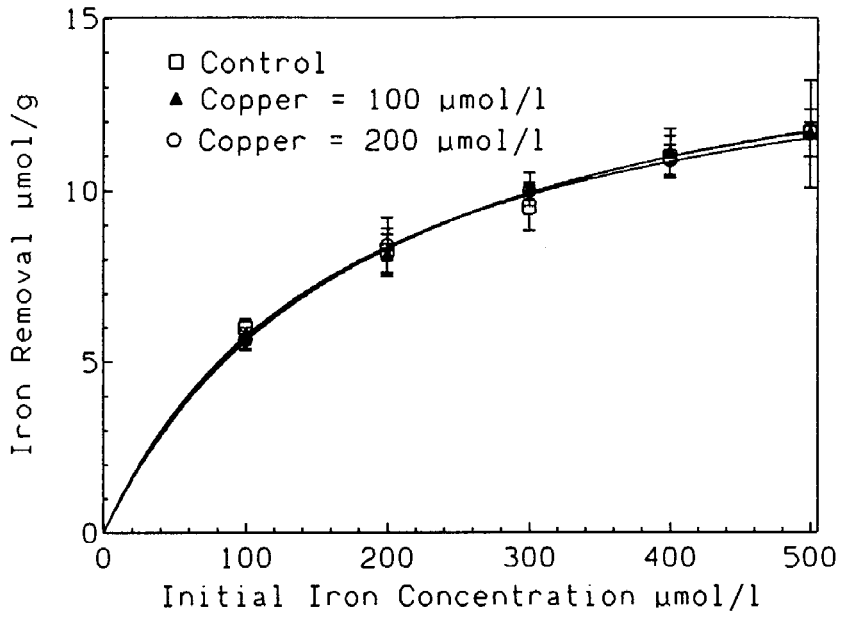

Figure 7.17: Sorption isotherms of initial iron concentrations and the effect on the iron removal with the addition of copper by brewer's *Saccharomyces cerevisiae* (n=6, ± SD). The incubation time was 30 min and the yeast concentration 10 mg/mℓ. Copper concentrations were 0, 100 and 200 µmol/ℓ.

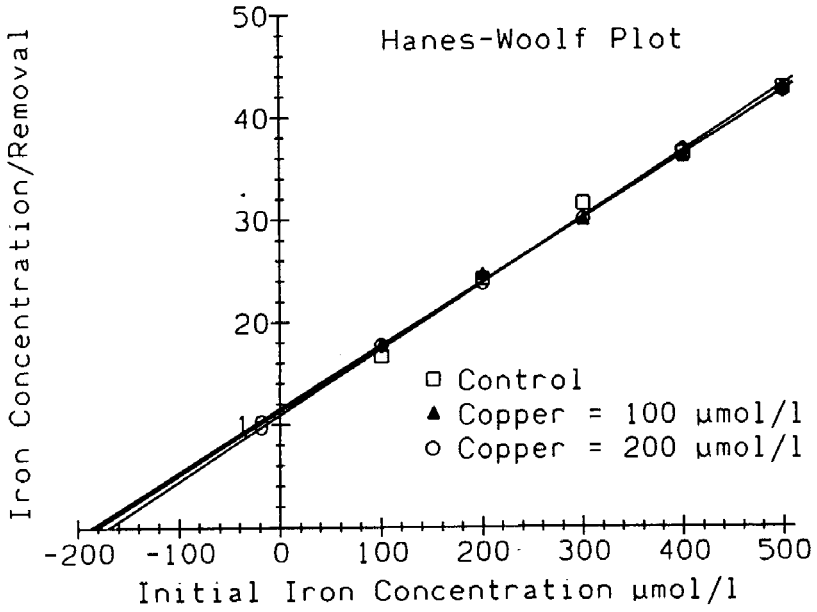

Figure 7.18: Hanes-Woolf plots showing the effect of copper on the removal of iron. The B_{max} and Kd of iron with no copper present were 15.5 µmol/g and 174 µmol/ℓ respectively, in the presence of 100 µmol/ℓ copper the B_{max}^{app} was 15.7 µmol/g and the Kd^{app} 188 µmol/ℓ and in the presence of 200 µmol/ℓ copper B_{max}^{app} was 16.1 µmol/g and the Kd^{app} was 182 µmol/ℓ.

A transmission electron micrograph of a cell exposed to a solution of iron and copper is shown in Figure 7.19. The transmission electron micrographs exhibited electron dense regions throughout the cytosol and the cell wall region. The ratio of copper to iron atomic percentages from EDXA of the cell cytoplasm consistently indicated a higher intracellular copper atomic percentage ratio. The copper to iron ratio ranged with different cells from 2:1 to as high as 5:1. Analysis of the cell wall region had a copper to iron ratio of 5:6. The EDXA spectra from the copper and iron investigation are shown in Figure 7.20 and 7.21.

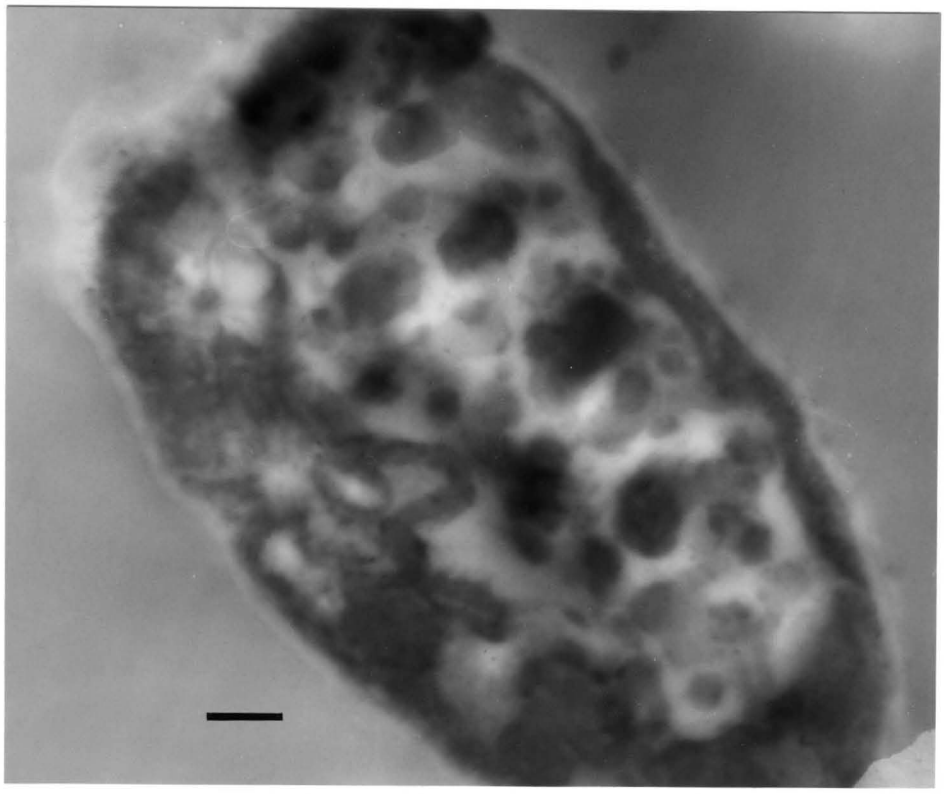

Figure 7.19: A TEM micrograph of a cross section of a *S. cerevisiae* cell after exposure to a mixed solution of copper and iron. The presence of metals in the electron dense areas were confirmed using EDXA. Scale bar = 0.5 μm.

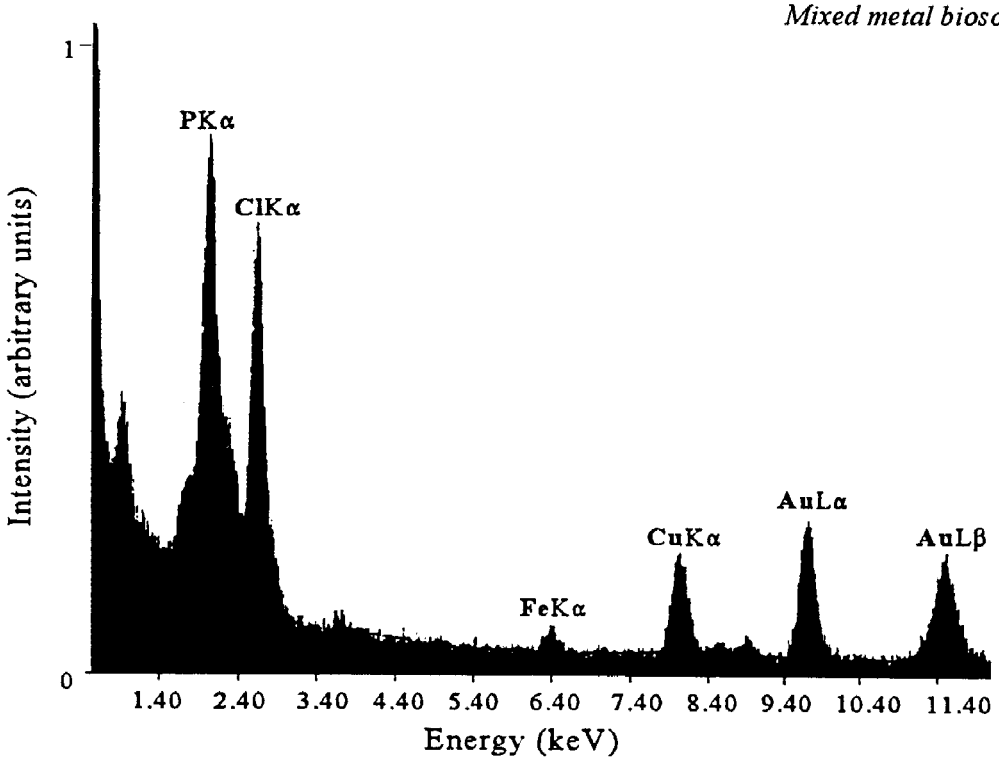

Figure 7.20: EDXA spectrum obtained from the cytoplasm of a cell of *Saccharomyces cerevisiae* exposed to 200μmoles/ℓ of copper and iron. Major identifiable peaks are P (2.01 keV), Cl (2.63 keV), Ca (3.75 keV), Fe (6.43 keV) and Cu (8.08 keV). The Au peaks are due to the supporting gold grids.

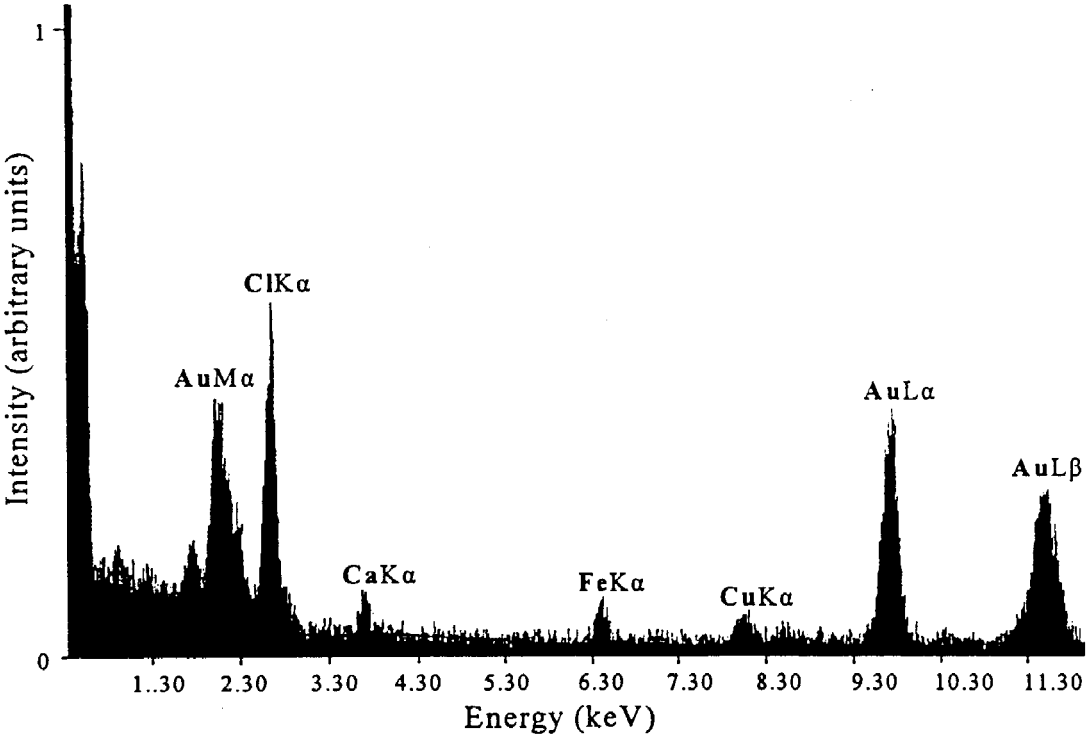

Figure 7.21: EDXA spectrum obtained from the margin (wall) of a cell of *Saccharomyces cerevisiae* exposed to 200μmoles/ℓ of copper and iron. Major identifiable peaks are Cl (2.63 keV), Ca (3.75 keV) and Fe (6.43 keV) and Cu (8.08 keV). The Au peaks are due to the supporting gold grids.

7.3.4 Bioremediation of metals from mine effluent

The effluent mine pH was ≤ 2. The initial metal concentrations were copper 21.3 μmol/ℓ, lead 7.3 μmol/ℓ, zinc 39.2 μmol/ℓ and iron 1078 μmol/ℓ. Removal of the metals at pH 2 and 3 was negligible. The effluent pH was adjusted to 4 which led to a certain amount of metal precipitation. Metal concentrations of the control experimental samples after precipitation were; 15.3 μmol/ℓ, lead 4.9 μmol/ℓ, zinc 37.7 μmol/ℓ and iron 460.0 μmol/ℓ. Bioremediation of the mine effluent in batch reactors is presented in Figure 7.22. At a yeast a concentration of 10 mg/mℓ, copper was decreased by 96.6 % to 0.6 μmol/ℓ, lead was decreased by 25.4 % to 3.6 μmol/ℓ and iron was decreased by 42.3 % to 262 μmol/ℓ. Zinc removal was ≤ 2%. Increasing the yeast concentration to 20 mg/mℓ increased lead removal to 36 % but had a negligible effect on removal of the other metals.

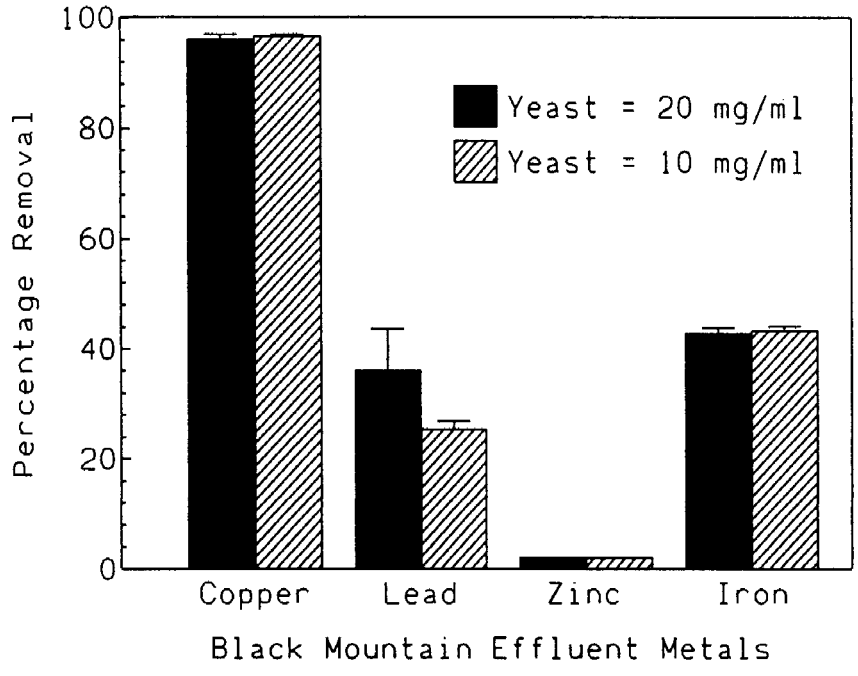

Figure 7.22: Percentage removal of metal from Black Mountain mine effluent in batch reactors (n=4, ± SD). The pH of effluent was adjusted to pH 4 with NaOH. The initial concentrations at pH 4 were copper 15.3 μmol/ℓ, lead 4.9 μmol/ℓ, zinc 37.7 μmol/ℓ and iron 460.0 μmol/ℓ. The yeast concentration was 10 and 20 mg/mℓ.

7.4 DISCUSSION

7.4.1 Copper and lead binary bioaccumulation

7.4.1.1 Effect of lead on copper removal

The maximum removal of copper from a single-ion solution by brewer's *S. cerevisiae* was 25.3 μmol/g, while it was 25.0 μmol/g in the presence of 100 μmol/ℓ lead and 25.5 μmol/g in the presence of 200 μmol/ℓ lead. The dissociation constants increased with increasing lead from 152 μmol/ℓ, with no lead, to 242 μmol/ℓ at an initial lead concentration of 200 μmol/ℓ. The consistent maximum removal rates and increasing Kd indicate a competitive interaction of the metals for binding sites (Gadd and Lawrence, 1996). At sufficiently high copper concentrations the lead was displaced with maximum copper removal not being affected at saturation.

7.4.1.2 Effect of copper on lead removal

Lead removal was significantly decreased in the presence of copper and, therefore, lower initial concentrations of copper were used as the secondary metal. The maximum uptake capacity of lead by the brewer's *S. cerevisiae* was decreased from 19.2 μmol/g in single-ion batch reactors to 14.0 μmol/g at an initial copper concentration of 50 μmol/ℓ. The Kd of lead removal decreased from 108 μmol/ℓ to 87 μmol/ℓ in the presence of 50 μmol/ℓ copper. The decrease in Kd indicates an initial sharper binding curve, possibly due to the effect of the competing copper on lead removal being less prominent at low metal concentrations due to sufficient surface binding sites for both the metals. The decrease in B_{max} and Kd is typical of uncompetitive inhibition which suggests a common uptake mechanism at a the lower copper concentration of 50 μmol/ℓ.

When the data of lead removal in the presence of 100 μmol/ℓ copper was transformed by Hanes-Woolf and Scatchard plot analysis, the results suggested that the inhibition may have been caused by a non-competitive mechanism. The B_{max}^{app} of lead was decreased further to 13.5 μmol/ℓ and the Kd concentration was increased to 118 μmol/ℓ. Non-

competitive inhibition suggests a second uptake mechanism at higher concentrations (Palmer, 1991). The decrease in lead uptake may be due to steric hindrance, as described by Tsezos *et al* (1997) for aluminum and uranium uptake by *R. arrhizus*, where the second metal uses a different uptake mechanism but influences binding of the primary metal.

7.4.1.3 Evaluation of copper and lead interference using absolute removal values

At an initial copper concentration of 200 μmol/ℓ, copper biosorption was 13.8 μmol/g while lead biosorption was 13.1 μmol/g at an initial lead concentration of 200 μmol/ℓ. At an initial copper concentration of 400 μmol/ℓ, copper biosorption was 17.4 μmol/g and at an initial lead concentration of 400 μmol/ℓ, lead biosorption was 15.3 μmol/g. In a mixed solution of copper and lead, at initial concentrations of 200 μmol/ℓ per metal (total initial concentration of 400 μmol/ℓ), copper removal was 11.5 μmol/g and lead removal was decreased to 6.6 μmol/g. Total metal removal from the mixed solution was 18.1 μmol/g which was higher than that of both lead and copper removal from single-ion batch reactors at an initial concentration of 400 μmol/ℓ.

This evaluation confirmed a competitive interaction between the metals and the antagonistic effect of copper on lead binding and, to a lesser extent, an antagonistic effect of lead on copper removal. The copper uptake in the mixed metal solution was decreased by 16.7 % and the lead uptake decreased by 49.6 % when compared to removal from single-ion solutions. This evaluation recognized the interaction between the metals but does not differentiate between the type of metal competition.

7.4.1.4 Energy dispersive X-ray analysis of copper and lead bioaccumulation

A relatively higher copper uptake from the mixed metal solution was confirmed by EDXA. The concentration ratios of copper to lead atomic percentages in the cell wall region indicated a higher copper to lead binding of up to 3:1 in the cells investigated. It would be reasonable to assume from the collective kinetic, absolute and EDXA generated data that copper has a higher affinity for surface binding sites than lead at the initial stage of biosorption. Metal deposition ratios in the cell cytoplasm were generally similar to those

in the cell wall, but in some cells the relative amount of lead was higher. This may indicate a intracellular uptake mechanism favouring lead. An alternate explanation could be that the cell sections analysed contained more lead, as it appeared from the transmission electron micrographs that the lead was deposited more uniformly around the cell cytoplasm, while the copper may have been localized to certain areas. Overall, the intracellular uptake of these two metals appears to be equal, with copper not being favoured, as may be expected from the kinetic investigations.

7.4.1.5 Copper and lead bioremediation from a mine effluent

The removal of copper and lead from the mine effluent followed a similar trend to the synthetic metal solutions, although comparisons are difficult due to the numerous co-contaminants. Copper removal was 96 % while the removal of lead was only 25.4 % efficient. The predicted removal of the two metals from a mixed synthetic metal solution at the concentrations of the effluent would be approximately 100 % for both metals.

7.4.2 Copper and zinc binary bioaccumulation

7.4.2.1 Effect of zinc on copper removal

The maximum uptake of copper by brewer's *S. cerevisiae* increased from 30.8 μmol/g in a single-ion system to 32.3 μmol/g in the presence of zinc at an initial concentration of 100 μmol/ℓ and increased to 34.5 μmol/g at an initial zinc concentration of 200 μmol/ℓ. The Kd values increased from 211 μmol/ℓ with no zinc in solution, 228 μmol/ℓ at 100 μmol/ℓ zinc and 264 μmol/ℓ at 200 μmol/ℓ zinc. The increase in the Kd values from the transformed binding isotherms appear to indicate some interaction between the copper and the zinc. The reasons for the increase in copper removal in the presence of zinc, and the mechanism involved, is unclear from the present investigation.

7.4.2.2 Effect of copper on zinc removal

As reported previously in section 6.4.2, zinc removal from solution by the brewer's yeast was low. In binary investigations, the removal of zinc was monitored in the presence of

low initial copper concentrations of 25 and 50 μmol/ℓ due to the high interference of copper on zinc removal. In the single-ion solutions the maximum removal of zinc was 13.1 μmol/g. At an initial copper concentration of 25 μmol/ℓ and 50 μmol/ℓ it decreased to 11.9 μmol/g and 10.6 μmol/g respectively. The Kd values increased with increasing copper in solution from 111 μmol/ℓ in the single-ion solution to 150 μmol/ℓ at copper 25 μmol/ℓ and 170 μmol/ℓ at an initial copper concentration of 50 μmol/ℓ. The inhibition of zinc uptake in the presence of copper at initial concentrations of 25 μmol/ℓ and 50 μmol/ℓ suggests a type of uncompetitive inhibition. The inhibition suggested that copper and zinc associate with the same binding sites and that the affinity of copper is substantially higher than zinc. Zinc bioaccumulation was effectively decreased by the copper at all concentrations investigated and selective binding of copper by the brewer's yeast was observed.

7.4.2.3 Evaluation of copper and zinc interference using absolute removal values

At initial copper concentrations of 200 μmol/ℓ and 400 μmol/ℓ, copper biosorption was 15.9 μmol/g and 20.3 μmol/g respectively. At initial zinc concentrations of 200 μmol/ℓ and 400 μmol/ℓ, zinc biosorption was 8.3 μmol/g and 10.9 μmol/g respectively. In a mixed solution of copper and zinc, at an initial concentration of 200 μmol/ℓ per metal, copper removal was 16.0 μmol/g and zinc removal was decreased to 5.1 μmol/g. Total metal removal from the mixed solution was 21.1 μmol/g which was higher than that of both the copper and zinc single-ion batch reactors at an initial concentration of 400 μmol/ℓ. Copper uptake in this investigation increased by 0.6 % in the presence of zinc suggesting the effect was largely non-interactive. Copper had an antagonistic effect on zinc uptake which decreased by 38.6 % in the presence of an equal initial concentration of copper.

De Carvalho *et al* (1995) also reported copper removal by immobilized *Ascophyllum nodosum* was preferred to zinc and that copper was less susceptible to zinc interference from mixed solutions. An increase in total removal was observed at final residual concentrations of 200 μmol/ℓ. Similar results were reported by Chong and Volesky

(1995) who observed a substantial decrease of zinc uptake by *Ascophyllum nodosum* in the presence of either copper or cadmium.

7.4.2.4 Energy dispersive X-ray analysis of copper and zinc bioaccumulation

The higher uptake of copper from the binary copper and zinc solutions was confirmed by EDXA. Initial metal binding was higher for copper with the concentration ratios of copper to zinc atomic percentages in the cell wall region of 2:1. The deposition ratios from X-ray analysis of the cell cytoplasm of copper to zinc was as high as 4:1. Both initial adsorption and intracellular uptake of the metals was preferential for copper. The combination of spectral data and the information from the kinetic studies suggest a common uptake mechanism for copper and zinc and that copper affinity for the biosorbent was higher, resulting in higher removal of copper relative to zinc.

7.4.2.5 Copper and zinc bioremediation from a mine effluent

The removal of copper and zinc from the mine effluent followed a similar trend. Zinc removal from the effluent was less than 2 % compared to the 96 % removal of copper. Zinc accumulation was possibly inhibited by other co-contaminants present in the effluent, as substantially higher zinc removal would have been expected in a equimolar synthetic binary solution.

7.4.3 Copper and iron binary bioaccumulation

7.4.3.1 Effect of iron on copper removal

The maximum uptake of copper by the brewer's *S. cerevisiae*, from a single-ion solution in batch reactors, was 25.4 μmol/g. In the mixed metal solutions, iron at an initial concentration of 100 μmol/ℓ, decreased the B_{max}^{app} of copper to 21.0 μmol/g and to 20.1 μmol/g at an initial iron concentration of 200 μmol/ℓ. The dissociation constant decreased with increasing iron from 215 μmol/ℓ, with no iron present, to 165 μmol/ℓ and 136 μmol/ℓ at initial iron concentrations of 100 μmol/ℓ and 200 μmol/ℓ respectively. The effect of iron on copper bioaccumulation, at the concentrations investigated, fits the uncompetitive

model with a decrease in B_{max} and Kd values. This model suggests a common binding site. At lower copper concentrations the effect of the iron is less pronounced as there are likely to be sufficient binding sites for both the metals and minimal interference is observed. The effect of iron on copper removal was more pronounced at higher copper concentrations and was reflected in a decrease in the maximum uptake capacity of copper. This suggests a higher affinity of iron for binding to the brewer's *S. cerevisiae.*

7.4.3.2 Effect of copper on iron removal

In the reciprocal study, the maximum uptake of iron from solution, in the single-ion batch reactors by the brewer's *S. cerevisiae*, was 15.5 μmol/g and increased slightly in the presence of 100 μmol/ℓ copper to 16.1 μmol/g and 16.0 μmol/g at an initial copper concentration of 200 μmol/ℓ. The dissociation constants increased slightly from 174 μmol/ℓ in a single-ion situation, 188 μmol/ℓ in the presence of 100 μmol/ℓ copper and 182 μmol/ℓ in the presence of 200 μmol/ℓ. Although copper uptake was higher than iron, copper did not interfere with the bioaccumulation of iron. The slight increase in the dissociation constant shows a limited interaction between the metals for bioaccumulation. The kinetic data suggests a low level of competition between the metals. The B_{max} remained relatively constant with a slight increase in the Kd values. Generally the iron removal was not affected by the presence of copper at the concentrations investigated.

7.4.3.3 Evaluation of copper and iron interference using absolute removal values

At an initial copper concentration of 200 μmol/ℓ and 400 μmol/ℓ, copper biosorption was 13.6 μmol/g and 17.6 μmol/g respectively. At an initial iron concentration of 200 μmol/ℓ and 400 μmol/ℓ, iron biosorption was 8.6 μmol/g and 11.3 μmol/g respectively. In a mixed solution of copper and iron, each at an initial concentration 200 μmol/ℓ, copper removal was 13.3 μmol/g and iron removal was decreased to 8.4 μmol/g. Total metal removal from the mixed solution was 21.7 μmol/g which was higher than that of both the copper and iron single-ion batch reactors of initial concentration of 400 μmol/ℓ. Copper uptake in this investigation decreased by 2.2 % in the presence of iron. Iron uptake was decreased by 2.3 % in the presence of an equal initial concentration of copper. This

evaluation suggests a slight antagonism between the metals for bioaccumulation to the yeast.

7.4.3.4 Energy dispersive X-ray analysis of copper and iron bioaccumulation

TEM micrographs showed electron dense regions which may be an indication of metal deposition. The concentration ratios of copper to iron atomic percentages from the EDXA spectra of the cell wall region ranged from 1:1 to 5:6. Analysis of the cell cytoplasm consistently gave a higher intracellular copper ratio. The copper to iron ratio varied from 2:1 to as high as 5:1. The collective data from the batch equilibrium and EDXA investigations suggest that the cell wall binds iron with a slightly higher affinity than copper. The much higher removal of copper is explained by the more efficient secondary intracellular uptake of copper into the cell cytoplasm. The bioaccumulation of copper into the cytoplasm would free binding sites on the cell wall and allow further adsorption of the metals until total saturation was reached. The EDXA data may therefore clarify the anomaly of the observed higher bioaccumulation of copper relative to iron, but the apparent higher affinity of iron binding relative to copper.

7.4.3.5 Copper and iron bioremediation from a mine effluent

Iron removal from the mine effluent was 42.3 %. The initial iron concentration was substantially higher than the other metals present in the effluent at 460 μmol/ℓ. Removal was 19.8 μmol/g which was substantially higher than that from the synthetic iron solutions of initial concentrations 400 (11.3 μmol/g) and 500 μmol/ℓ (11.6 μmol/g).

7.4.4 Other reported dual metal investigations

Reports on the bioaccumulation of metals from mixed metal solutions are relatively uncommon. Recently the importance of investigating dual metal systems has been recognized although reports are infrequent and the metal combinations specific to the interests of the particular researches. Gadd and Lawrence (1996) reported on the effects of magnesium on the uptake of manganese. Manganese was found to be transported into *S. cerevisiae* cells by two transport systems, a low concentration, high affinity system and

a higher concentration, low affinity system. At a low range between 25-1000 nmol/ℓ the mechanism was highly specific and even at 50 fold excesses of magnesium the uptake remained high at 72 %, thus maintaining cellular requirements. At low concentrations the effects of magnesium did not fit Michaelis-Menten inhibition models. At a manganese concentration range of 5 to 200 μmol/ℓ, the presence of 50 μmol/ℓ magnesium caused competitive inhibition. At 100 μmol/ℓ magnesium non-competitive inhibition was observed. The concentrations of the metals in solution are important considerations and influenced the inhibition of primary metal accumulation depending on bioaccumulation mechanisms.

Galun *et al* (1984) reported a 50 % inhibition of 100 ppm uranium uptake in the presence of 25 ppm ferric (Fe^{3+}) ion. Competitive biosorption studies between chromium (VI) and Fe (III) to *R. arrhizus* by Săg and Kutsal (1996b), found uptake was decreased by the presence of increasing concentrations of the second metal. Long-term chromium (VI) uptake in the presence of iron was greater than iron under the same conditions. The combined effect of the two metals on the *R. arrhizus* biomass was antagonistic. Cadmium uptake was reduced in the presence of iron at a final concentration of 1.5 mmol/ℓ per metal by 24 %. Iron uptake was more strongly suppressed by cadmium than vice versa. (Figueira *et al*, 1997). Săg and Kutsal (1996a) reported a partial non-competitive inhibition between copper and chromium (VI). Copper uptake was decreased in the presence of chromium (VI). Total uptake in the dual metal systems was higher than in single-ion biosorption systems due to better use of the fungal active sites because of the partial competition for adsorption of metal ions (Săg and Kutsal, 1996a). Tobin *et al* (1988) reported the presence of competing cations in solution to cause a wide degree of inhibition of metal uptake by *R. arrhizus*. Silver was almost completely displaced by equimolar uranium ions. Uranium uptake was relatively unaffected by a large molar excess of silver. Lanthanum had a similar effect on silver uptake. A 5-fold molar cadmium excess reduced silver uptake by 50 % while silver had a negligible effect on cadmium. The mechanism of biosorption from these mixed solutions was proposed as simple cation competition (Tobin, 1988).

Biosorption of metals from solutions containing more than two metals has also been reported. Interpretation of this data is complex and multi-mathematical approaches are being developed to describe and evaluate the performance of multi-metal systems (Volesky, 1995; Chong and Volesky, 1996). A four metal biosorption to *Chlorella vulgaris* containing initial concentrations of copper and lead of 5 mg/ℓ per metal, 0.5 mg/ℓ zinc and 2 mg/ℓ cadmium was investigated by Pascucci (1993). After 2.5 h the algae bound 70 % lead, 55 % copper, 45 % cadmium and 35 % of the zinc. The process was pH and biomass concentration dependant. Earlier work by Nakajima and Sakuguchi (1986) investigated the accumulation of metals by a wide range of microorganisms from solutions containing equal concentrations of manganese, cobalt, nickel, copper, zinc, cadmium, mercury, lead and uranium. Mercury, lead and uranium ions were readily accumulated by the microorganisms investigated, while manganese, cobalt, nickel, copper, zinc and cadmium accumulation was low. Cobalt from a single-ion solution was accumulated in large amounts by the same microorganisms. These authors reported a removal of 15 μmol/g copper, 111 μmol/g mercury, 62 μmol/g lead and 94 μmol/g uranium by *S. cerevisiae* from the mixed metal solution. The remaining 5 metals were not accumulated. They proposed selective bioaccumulation of metals due to interionic competition. Uranium removal increased from 49 μmol/g in a single-ion investigation to 99 μmol/g from the mixed metal solution. This increase was not explained.

Understanding the competition mechanism between metals is important in augmenting the knowledge in the development of applications of the bioremediation technology. The biosorption from mixed metal solutions is dependent on the characteristics of the particular competing metal species, pH, the metal concentrations, the biomass and the type of uptake mechanisms utilized by the biomass.

7.5 CONCLUSION

Combined batch reactor biosorption and EDXA investigations determined uptake characteristics from dual metal solutions by waste brewer's *Saccharomyces cerevisiae*.

Transformation of the initial rates of copper uptake suggested inhibition caused by lead was competitive. Lead uptake was decreased by copper and inhibition characteristics were dependent on copper concentration. Copper and lead deposits on the cell wall and in the cytoplasm were confirmed by EDXA. Copper was detected at higher ratios on the cell wall which suggested a higher affinity for initial binding.

The presence of zinc in solution had a synergistic effect on copper removal by the brewer's *Saccharomyces cerevisiae*. Zinc uptake was strongly inhibited in the presence of copper. The presence of copper and zinc on the cell wall and in the cell cytoplasm was confirmed by EDXA. The combined biosorption and spectral results suggested a common uptake mechanism, with copper having a substantially higher binding affinity.

Copper uptake by the brewer's *Saccharomyces cerevisiae* was higher than iron from single-ion solutions. Transformation of the initial rates of copper uptake suggested inhibition caused by iron in mixed metal solutions was uncompetitive, and that iron was not inhibited by copper. The combined biosorption and spectral results showed a higher affinity of iron binding to the cell wall, however, intracellular uptake was higher for copper.

The metal removal from a mine effluent complemented the results from synthetic dual metal solutions, however, the removal efficiency remained low, with the exception of copper. Iron removal was satisfactory, considering the relatively high initial concentration. Lead and zinc uptake was possibly decreased due to the presence of high levels of co-contaminants such as; calcium, magnesium and sulphate.

The combined investigations of mixed metal biosorption and EDXA could provide valuable insight into the elucidation of metal biosorption mechanisms and provide the impetus required for the application of the technology.

CHAPTER VIII

8 GENERAL DISCUSSION

Population growth, escalating urbanisation and industrialisation are placing ever increasing demands on South Africa's limited water resources. The careful management and conservation of these water resources is essential for the long-term provision of quality water for both human and industrial consumption. Metal contamination of local water supplies from urban run-off and industrial pollution such as battery manufacture and mining, have a negative affect on the environment and may cause unacceptable risks to human health. Conventional purification of water mostly involves chemical treatment and settling of the precipitated metal. This method is expensive, is not environmentally sound and has the added disadvantage of generating large quantities of sludge. Research has recently focused on the development of more effective waste water purification systems such as bioremediation. Microbial biomass has the ability to accumulate toxic and valuable metals from aqueous solutions. This project considered the use of the yeast *Saccharomyces cerevisiae* as a biosorbent for the removal and recovery of a range of metals from metal contaminated waters. *S. cerevisiae* as a biosorbent has the potential to provide a cost effective, selective and highly efficient purification system.

Initial studies focused on metal accumulation by an immobilized baker's *S. cerevisiae* biosorbent. The yeast was immobilized mainly to facilitate liquid/solid separation. Parameters affecting metal uptake were investigated, these included metal concentration, time and solution pH. The metals investigated included copper, zinc, nickel, cobalt, cadmium and chromium. They were chosen because they are frequently encountered in industrial waste waters. Gold bioaccumulation was investigated because of the value of the metal.

Metal uptake was rapid. Gold and cobalt reached saturation within 5 min of contact with the biosorbent in batch reactors. Copper, zinc, nickel, cadmium and chromium reached saturation within 30 min of contact. Metal accumulation was pH dependent and was generally unaffected at a solution pH $\geq$ 4, and was substantially decreased at pH $\leq$ 2. The exception was gold which

was preferentially accumulated at a solution pH of 2. The immobilized baker's yeast accumulated metals with maximum binding capacities in the order of gold > cadmium > cobalt > zinc > copper > chromium > nickel.

Desorption of bioaccumulated metal was investigated in batch reactors. The ability to recover metals from the biosorbent has important environmental and economic implications. Successful metal recovery would allow total removal from the biosphere and metal recycling. The biosorbent may also be recycled for continuous use. These two factors would considerably lower the cost of a bioremediation system. A rapid method for the assessment of metal recovery from the immobilized *S. cerevisiae* was developed. Copper recovery of ≥ 80 % was attained by decreasing the solution pH to ≤ 2 with the addition of HCl, H_2SO_4 or HNO_3. Adsorption-desorption cycles, of up to 8 cycles, had no apparent adverse effect on the uptake or recovery of metal from batch reactors. No major morphological changes or damage to cells used in adsorption-desorption studies were observed under transmission electron microscopy investigations.

Biosorption columns were investigated as bioreactors due to their application potential. The advantages of these reactors include; ease of metal application, liquid/solid separation, easy recovery of bound metal, concentration of metal and convenient regeneration of the biomass. The metals investigated were effectively removed from solution. Columns were saturated in the direction of solution flow. At a saturation threshold, metal uptake declined rapidly. Most metals investigated were desorbed from the columns by eluting with 0.1 M HCl. Recoveries of copper, cobalt and cadmium were as high as 100 %. Desorbed copper, zinc, cadmium, nickel and cobalt were concentrated in 10 to 15 mℓ of eluent, representing up to a 40 fold decrease in solution volume. From an application perspective, a high concentration factor would decrease the effluent volume and make it more manageable. This would be advantageous for both metal and biosorbent recycling.

The columns were reconditioned and the metals reapplied. Cadmium, nickel and zinc uptake increased with the second application to the columns. The increased uptake was possibly due to the exposure of additional binding sites during the reconditioning procedure. Initial accumulation

of gold and chromium was 42.2 μmol/g and 28.6 μmol/g, however, due to the low recoveries of these two metals, a second application was not investigated. Copper was applied to a single column for 8 consecutive adsorption-desorption cycles. The uptake from the first cycle was 31.3 μmol/g. Uptake progressively increased from the fourth cycle to 47.8 μmol/g at cycle 7. As observed in the batch reactors, copper removal and recovery was not adversely affected with repeated adsorption-desorption applications.

The potential for selective metal recovery was demonstrated using biosorption columns. Binary metal solutions of copper and zinc; copper and cobalt; and copper and cadmium were applied to two columns in series. Copper was accumulated and recovered most efficiently from the first column in the series. Zinc, cobalt and cadmium were displaced to the second column. Copper bound preferentially to zinc at a ratio of 6:1. Copper bound preferentially to cobalt at a ratio of 4:1. Cadmium was only displaced at a ratio of 2:1. The system would require further optimization to obtain total separation of the metals. Biosorbent reusability and selective metal recovery are important considerations for the development of an applied bioremediation system.

The treatment of industrial effluents is problematic due to the complex character of these waste waters. Industrial effluents typically contain more than one metal species and numerous other co-contaminants. Although the prospects of bioremediation are good, the successful transfer of the technology from the laboratory to an industrial application has yet to be realized. Bioremediation of a mine effluent containing copper, zinc, lead and iron was investigated. The removal of the metals was most effective at pH 4. The combined strategy of pH adjustment and bioremediation decreased the copper concentration by 92.5 %, lead was decreased by 90 % and zinc was decreased 60 %. Iron was mostly precipitated from solution at pH $\geq$ 4. A visit to the mine led to the identification of a potential bioremediation site. The site was an ageing pond used for chemical treatment of the waste water. The conditions, such as; pH, water volume and metal concentrations, were potentially more conducive to a bioremediation process. The investigation indicated a possible application of the biomass as a supplement to a chemical remediation process.

Due the financial implications of using the commercially available baker's *S. cerevisiae*, it was

decided to investigate the metal uptake potential of waste brewer's yeast. The waste yeast was obtained after several fermentations from a local brewery. It was generally in a stressed physiological condition which differed from batch to batch. The metal removal capacities were initially highly inconsistent. A pre-adsorption yeast condition protocol was developed which increased metal uptake up to 100 % and enhanced reproducibility. Synthetic solutions containing the metals copper, zinc, lead and iron were investigated as these were the target metals in the mine effluent under investigation.

Metal removal from solution was rapid with saturation being reached within 15 min. As with the immobilized baker's yeast, the metal removal was pH dependant. The metals were efficiently removed from solution at pH $\geq$ 4. Uptake was substantially inhibited at pH$\leq$ 3. The waste brewer's yeast accumulated metals with maximum binding capacities in the order of copper (25.4 μmol/g) > lead (19.4 μmol/g) > iron (15.6 μmol/g) > zinc (12.5 μmol/g). No correlation between cell physiology and metal uptake was observed. Uptake of the four metals was confirmed by energy dispersive X-ray microanalysis. Metal deposits were detected in the cell wall region and in the cell cytoplasm.

The presence of two or more metals in solution causes changes to the solution chemistry and can effect the biochemical processes of the biosorbent. The metals compete for bioaccumulation by the biomass. The interference of lead, zinc and iron on copper uptake by the waste brewer's yeast, and the interference of copper on the uptake of lead, zinc and iron was investigated. Maximum copper uptake was not decreased in the presence of lead. The B_{max} remained constant at approximately 25 μmol/g. The dissociation constants increased with increasing lead concentrations, which suggested a competitive inhibition. Lead bioaccumulation was significantly decreased in the presence of copper. The type of inhibition displayed was dependent on the initial copper concentrations. Copper and lead deposits were confirmed in the cell wall regions and cytoplasms of the yeast cells.

Zinc had a slight synergistic effect on copper uptake. The copper B_{max} increased from 30.8 μmol/g in a single-ion system to 34.5 μmol/g in the presence of 200 μmol/ℓ of zinc. Zinc uptake

was severely inhibited in the presence of copper. Both the maximum uptake and dissociation constant values were decreased in the presence of copper, which suggested an uncompetitive inhibition. This type of inhibition suggests common binding sites. The affinity of copper was substantially higher than zinc. The presence of higher levels of copper than zinc in the yeast cells was confirmed by energy dispersive microanalysis.

Copper uptake was decreased in the presence of iron, with the copper B_{max} being decreased from 25.4 μmol/g in a single-ion system to 20.1 μmol/g in the presence of 200 μmol/ℓ iron. Iron B_{max} values remained constant at 16.0 μmol/g. Combined biosorption and EDXA results suggested the iron bound at a higher affinity than copper to the cell wall. Total copper removal was higher as larger quantities of copper were deposited in the cell cytoplasm.

Metal removal from the Plaatjiesvlei effluent by free cell suspensions of the waste brewer's yeast was investigated. At pH 4, copper levels were decreased by 96 %, iron by 42 %, lead 25 % and zinc 2 %.

The current approach to bioremediaton relies largely on bench-scale laboratory results. For the technology to progress, successful transfer to industrial application is essential. Industrial conditions are site specific. Knowledge of the industrial process and composition of the effluent, could lead to a consistent, manageable waste water which could be more conducive to biological treatment. Ongoing investigations in our laboratory include the investigation of a local battery manufacture effluent. The close proximity of the breweries to the effluent production site decreases transport costs, and enhances the possibility of applying the waste product of one industry to the treatment a second industry's effluent. Waste brewer's yeast is presently a cheap source of biomass in South Africa, and could provide the basis for an innovative waste water purification system.

APPENDICES

APPENDIX 1

Table A.1 : Polyacrylamide gel metal removal controls.

Metal	Initial concentration	Removal μmol/g	Removal Percentage
Cobalt chloride	200 μmol/ℓ	0.072 ± 0.036[a]	3.13 ± 1.57[a]
Chromium chloride	200 μmol/ℓ	0.127 ± 0.020[a]	6.02 ± 0.97[a]
Copper chloride	200 μmol/ℓ	0.332 ± 0.033[a]	15.76 ± 1.55[a]
Cadmium chloride	200 μmol/ℓ	0.007 ± 0.017[a]	0.44 ± 0.98[a]
Nickel chloride	200 μmol/ℓ	0.175 ± 0.026[a]	7.52 ± 1.12[a]
Zinc chloride	200 μmol/ℓ	0 ± 0	0 ± 0
Copper sulphate	200 μmol/ℓ	0.368 ± 0.033[a]	18.16 ± 1.64[a]
Zinc sulphate	200 μmol/ℓ	0.009 ± 0.210[a]	0.50 ± 1.11[a]
Iron sulphate	200 μmol/ℓ	0.290 ± 0.042[a]	16.08 ± 2.35[a]
Lead nitrate	200 μmol/ℓ	0.485 ± 0.028[a]	24.47 ± 1.41[a]
Gold	200 μmol/ℓ	0.109 ± 0.018[a]	4.70 ± 0.80[a]
Mine effluent copper	18 μmol/ℓ	0.007 ± 0.007[a]	3.83 ± 4.13[a]
Mine effluent zinc	33 μmol/ℓ	0.013 ± 0.008[a]	3.86 ± 2.41[a]
Mine effluent iron	315 μmol/ℓ	0.149 ± 0.034[a]	4.70 ± 1.11[a]
Mine effluent lead	4 μmol/ℓ	0.001 ± 0.001[a]	2.63 ± 3.63[a]

a - Mean ± SD of 5 determinations

APPENDIX 2:

Physiology investigations

Discrepancies of yeast viability may occur between different techniques due to the different criteria used to assess cell death. The technique used for viability should be relevant to the particular investigation and should always be referenced.

A.2.1 Yeast viability methylene blue staining

Methylene blue staining is used to determine the ratio of dead to alive yeast cells. Live cells are refractory to the dye while the dead cells readily take up the stain. Live cells contain reducing compounds such as NADH which reduce methylene blue to a colourless form. Two drops of the yeast cell solution on a slide to which 2 drops of methylene blue are added and mixed well. The cells are counted on a haemocytometer after standing for 1 min.

Percentage viability = (number colourless cells/total cells) x 100

(Hough, 1971 and 1984)

A.2.2 Determination of protease activity:

The assay measures excretion products due the autolysis of dead yeast cells. Casein labelled with [N-(resonifin-Δ-carbonyl piperidine-4-carbonic acid-N-hydroxysuccinimide ester] is hydrolysed by proteases into peptides which retain the label and can be assayed photospectrometrically at 574 nm. Unhydrolysed casein is removed by trichloroacetic acid (TCA) precipitation.

The sample yeast is equilibrated to the temperature at which the pH meter is calibrated and is degassed before pH measurement. The protease assay is only accurate at pH values above 4.0 and below 4.9. The yeast slurry is centrifuged at 10 000 x g for 5 min. The supernatant is assayed for protease activity. Pasteurized beer (25 mℓ) is heated at 100° C for 5 min, centrifuged

as above and used as a control sample.

Add 50 $\mu\ell$ of labelled casein substrate, 50 $\mu\ell$ 0.2 M Tris-HCl buffer (pH 7.8) and 100 $\mu\ell$ sample to a 1.5 mℓ eppendorf tube. Incubate for 1 h at 37° C after gentle mixing. The reaction is stopped by adding 480 $\mu\ell$ TCA which precipitates unhydrolysed casein. Incubate at 10 min for 37° C. Centrifuge at 10 000 x g for 5 min. The supernatant (400 $\mu\ell$) is added to 600$\mu\ell$ 0.5 M Tris-HCl assay buffer (pH 8.8), mixed and read at 574 nm. Protease activity is expressed as delta absorbance (ΔAbs): ΔAbs = sample absorbances - blank absorbances. The ΔAbs of pitching yeast (viability above 95 %) should not be above 0.01.

Methodology supplied by the South African Breweries, Beer division brewing manual(1996), Analytical methods, vol. 10, section 7.31 from Mochaba F. *et al* (1993).

REFERENCES

Al-Asheh S. and Duvnjak Z. (1995) Adsorption of copper and chromium by *Aspergillus carbonarius*, *Biotechnol. Prog.*, **11**, 638-642.

Akthar N., Sastry S., Mohan K. and Mohan M. (1995) Biosorption of silver ions by processed *Aspergillus niger* biomass, *Biotechnol. Lett.*, **17**, 551-556.

Anderson G.J., Lesuisse E., Dancis A., Roman D.G., Labbe P. and Klausner R.D. (1992) Ferric iron reduction and iron assimilation in *Saccharomyces cerevisiae*, *J. Inorgan. Biochem.*, **47**, 249-255.

Ashida J. (1965) Adaption of fungi to metal toxicity, *Annual review of Plant Pathology*, **3**, 153-174.

Avery S.V., Codd G.A. and Gadd G.M. (1992) Replacement of cellular potassium by caesium in *Chlorella emersonii*: Differential sensitivity of photoautotrophic and chemoheterotrophic growth, *J. Gen. Microbiol.*, **138**, 69-76.

Babich H. and Stotzky G. (1980) Effects of cadmium on the biota: Influence of environmental factors, *Adv. Appl. Microbiol.*, **25**, 55-117.

Babich H. and Stotzky G. (1985) Heavy metal toxicity to microbe-mediated ecologic processes: A review and potential application to regulatory policies, *Environ. Res.*, **36**, 111-137.

Babu S.R.V., Wolfian J.H. and Chapatwala K.D. (1993) Degradation of inorganic cyanides by immobilized *Pseudomonas putida* cells. In: *Proc. Biohydrometallurgy Symp. Vol II, Biohydrometallurgical technologies*, Torma A.E., Apel M.L., Brierley C.L. (eds), USA.

Bartnicki-Garcia S. and McMurrough I. (1971) Biochemistry of morphogenesis. In: *The Yeasts, Physiology and biochemistry of yeasts*, vol 2, Rose A.H. and Harrison J.S. (eds), Academic Press, London, 441-492.

Basnakova G. and Macaskie L.E. (1997) Microbially enhanced chemisorption of nickel into biologically synthesized hydrogen uranyl phosphate: A novel system for the removal and recovery of metals from aqueous solutions, *Biotechnol. Bioeng.*, **54**, 319-328.

Baxter D.A. (1996) Philosophies of desludging management at Rand Water, *Proc. biennial conference of the Water Institute of Southern Africa*, **1**, paper 4, Port Elizabeth.

Belde P.J.M., Kessels B.G.F., Moelans J.M. and Borst-Pauwels G.W.F.H. (1988) Cd^{2+} uptake, Cd^{2+} binding and loss of cell K^+ by a Cd-sensitive and a Cd-resistant strain of *Saccharomyces cerevisiae*, *FEMS Microbiol. Lett.*, **49**, 493-498.

Bender J., Lee R.F. and Phillips P. (1995) Uptake and transformation of metals and metalloids by microbial mats and their use in bioremediation, *J. Ind. Microbiol.*, **14**, 113-118.

Beveridge T.J. and Murray R.G.E. (1976) Uptake and retention of metals by cell walls of *Bacillus subtilis*, *J. Bacteriol.*, **127**, 1502-1518.

Beveridge T.J. (1986) The immobilization of soluble metals by bacterial cell walls, *Biotechnol. Bioeng. Symp.*, **16**, 127-139.

Beyersmann D. and Hartwig A. (1992) The genetic toxicology of cobalt, *Toxicol. Appl. Pharmacol.*, **115**, 137-145.

Bhagat R. and Srivastava S. (1994) Effect of zinc on morphology and ultrastructure of *Pseudomonas stutzeri* RS34, *J. Gen. Appl. Microbiol.*, **40**, 265-270.

Bianchi M.E., Carbone M.L, Lucchini G. and Magni G.E. (1981) Mutants resistant to manganese in *Saccharomyces cerevisiae*, *Current Genetics*, **4**, 215-220.

Blackburn J.W. and Hafker W.R. (1993) The impact of biochemistry, bioavailability and bioactivity on the selection of bioremediation techniques, *TIBTECH*, **11**, 328-333.

Blackwell K.J., Singleton I. and Tobin J.M.(1995) Metal cation uptake by yeast: A review, *Appl. Microbiol. Biotechnol.*, **43**, 579-584.

Borbolla M. and Peña A. (1980) Some characteristics of Ca^{2+} uptake by yeast cells, *J. Membrane Biol.*, **54**, 149-156.

Bordons A. and Jofre J. (1987) Extracellular adsorption of nickel by a strain of *Pseudomonas sp.*, *Enzyme Microb. Technol.*, **9**, 709-713.

Borst-Pauwels G.W.F.H. (1981) Ion transport in yeast, *Biochim. Biophys. Acta.*, **650**, 88-127.

Borst-Pauwels G.W.F.H. (1993) Kinetical parameters of monovalent cation uptake in yeast calculated on accounting for the mutual interaction of cation uptake and membrane potential, *Biochim. Biophys. Acta.*, **1152**, 201-206.

Brady D. and Duncan J.R. (1994a) Bioaccumulation of metal cations by *Saccharomyces cerevisiae*, *Appl. Microbiol. Biotechnol.*, **41**, 149-154.

Brady D., Stoll A. and Duncan J.R. (1994b) Biosorption of heavy metal cations by non-viable yeast biomass, *Environ. Technol.*, **15**, 429-438.

Brady D., Letebele B., Duncan J.R. and Rose P.D. (1994c) Bioaccumulation of metals by *Scenedesmus*, *Selenastrum* and *Chlorella* algae, *Water SA*, **20**, 213-218.

Brady D. and Duncan J.R. (1994d) Binding of heavy metals by the cell walls of *Saccharomyces cerevisiae*, *Enzyme. Microb. Technol.*, **16**, 633-638.

Brady D. and Duncan J.R. (1994e) Cation loss during accumulation of heavy metal cations by *Saccharomyces cerevisiae*, *Biotechnol. Lett.*, **16**, 543-548.

Brady D., Glaum D. and Duncan J.R. (1994f) Copper tolerance in *Saccharomyces cerevisiae*, *Lett. Appl. Microbiol.*, **18**, 245-250.

Brady D., Stoll A., Starke L. and Duncan J.R. (1994) Chemical and enzymatic extraction of heavy metal binding polymers from isolated cell walls of *S. cerevisiae*, *Biotechnol. Bioeng.*, **44**, 297-302.

Brierley J.A. (1987) Microbial removal of metals from industrial wastewaters, *Water and Wastewater Int.*, **2**, 31-35, 54.

Brierley C.L. and Brierley J.A. (1993) Immobilisation of biomass for industrial application of biosorption. In: *Proc. Intl. Biohydrometallurgy Symp.*, *Biohydrometallurgical Technologies II*, Torma A.E., Apel M.L. and Brierley C.L. (Eds), USA, 35-44.

Bryers J.D. (1993) The biotechnology interfaces, *J. Appl. Bacteriol. Symp. Supplement*, **74**, 98S-109S.

Bux F., Swalaha F.M. and Kasan H.C. (1995) Assessment of acids as desorbents of metal ions bound to sludge surfaces, *Water SA*, **21**, 319-324.

Cabral J.P.S. (1989) Induction of potassium efflux by cupric ions *Pseudomonas syringae* ATCC 12271 and its correlation with cell viability, *Microbios*, **60**, 141-150.

Cabral J.P.S. (1990) Cupric ions induce an efflux of potassium and low molecular mass metabolites in *Pseudomonas syringae*, *FEMS Microbiol. Lett.*, **72**, 109-112.

Cabral J.P.S. (1992) Starved *Pseudomonas syringae* cells release strong Cu^{2+}-complexing compounds, *Chemical Speciation and Bioavailability*, **4**, 105-107.

Carenza M. and Veronese F.M. (1994) Entrapment of biomolecules into hydrogels obtained by radiation-induced polymerization, *J. Controlled Release*, **29**, 187-193.

Chamy R., Nuñez M.J. and Lema J.M. (1990) Optimization of the hardening treatment of *S. cerevisiae* bioparticles, *Enzyme Microb. Technol.*, **12**, 749-754.

Chatterjee S., Asthana R.K., Tripathi A.K. and Singh S.P. (1996) Metal removal by selected sorbents, *Process Biochem.*, **31**, 457-462.

Chen P. and Ting Y.P. (1995) Effect of metal uptake on the electrokinetic properties of *Saccharomyces cerevisiae*, *Biotechnol. Lett.*, **17**, 107-112.

Chibata I., Tosa T. and Sato T. (1974) Immobilized aspartase-containing microbial cells: Preparation and enzymatic properties, *Appl. Microbiol.*, **27**, 878-885.

Chibata I., Tosa T. and Takata I. (1983) Continuous production of L-malic acid by immobilized cells, *TIBTECH*, **1**, 9-11.

Chien N.K. and Sofer S.S. (1985) Flow rate and bead size as critical parameters for immobilized-yeast reactors, *Enzyme Microb. Technol.*, **7**, 538-542.

Chmielowski J. and Klapcińska B. (1986) Bioaccumulation of germanium by *Pseudomonas putida* in the presence of two selected substrates, *Appl. Environ. Microbiol.*, **51**, 1099-1103.

Cho D., Lee S., Park S. and Chung A. (1994) Studies on the biosorption of heavy metals onto *Chlorella vulgaris*, *J. Environ. Sci. Health*, **A29**, 389-409.

Chong K.H. and Volesky B. (1995) Description of two-metal biosorption equilibria by langmuir-type models, *Biotech. Bioeng.*, **47**, 451-460.

Chong K.H. and Volesky B. (1996) Metal biosorption equilibria in ternary system, *Biotechnol. Bioeng.*, **49**, 629-638.

Conklin D.S., McMaster J.A., Culbertson M.R. and Kung C. (1992) COT1, a gene involved in cobalt accumulation in *Saccharomyces cerevisiae*, *Mol. Cell Biol.*, **12**, 3678-3688.

Conklin D.S., Culbertson M.R. and Kung C. (1994) Interactions between gene products involved in divalent cation transport in *Saccharomyces cerevisiae*, *Mol. Gen. Genet.*, **244**, 303-311.

Cotoras D., Viedma P. and Pimentel J. (1993) Biosorption of metals by attached bacterial cells in a packed-bed bioreactor. In: *Proc. Intl. Biohydrometallurgy Symp.*, *Biohydrometallurgical Technologies II*, Torma A.E., Apel M.L. and Brierley C.L. (Eds), USA, 103-110.

Crist R.H., Martin J.R., Carr D., Watson J.R., Clarke J. and Crist D.R. (1994a) Interaction of metals and protons with alga. 4. Ion exchange vs adsorption models and a reassessment of scatchard plots; ion-exchange rates and equilibria compared with calcium alginate, *Environ. Sci. Technol.*, **28**, 1859-1866.

Crist D.R., Crist R.H., Martin J.R. and Watson J.R. (1994b) Ion exchange systems in proton-metal reactions with algal cell walls, *FEMS Microbiol. Reviews*, **14**, 309-314.

Cross R.H.M. (1989) A reliable epoxy resin mixture and its application in routine biological transmission electron microscopy, *Micron and Microscopia Acta*, **20**, 1-7.

Davidova E.G. and Kasparova S.G. (1993a) Sorption of heavy metals by yeast cell walls, *Mikrobiologiya*, **61**, 838-842.

Davidova E.G. and Kasparova S.G. (1993b) Adsorption of metals by yeast cell walls, *Mikrobiologiya*, **61**, 1018-1022.

De Carvalhor R.P., Chong K.H. and Volesky B. (1995) Evaluation of the Cd, Cu, and Zn biosorption in two-metal systems using an algal biosorbent, *Biotechnol. Prog.*, **11**, 39-44.

De Rome L. and Gadd G.M. (1987) Measurement of copper uptake in *Saccharomyces cerevisiae* using a Cu^{2+} -selective electrode, *FEMS Microbiol. Lett.*, **43**, 283-287.

De Rome L. and Gadd G.M. (1991) Use of pelleted and immobilized yeast and fungal biomass for heavy metal and radionuclide recovery, *J. Indust. Microbiol.*, **7**, 97-104.

Dressler C., Kües U., Nies D.H. and Friedrich B. (1991) Determinants encoding resistance to several heavy metals in newly isolated copper-resistant bacteria, *Appl. Environ. Microbiol.*, **57**, 3079-3085.

Eilam Y. and Chernichovsky D. (1987) Uptake of Ca^{2+} driven by the membrane potential in energy-depleted yeast cells, *J. Gen. Microbiol.*, **133**, 1641-1649.

Engl A. and Kunz B. (1995) Biosorption of heavy metals by *Saccharomyces cerevisiae*: Effects of nutrient conditions, *J. Chem. Tech. Biotechnol.*, **63**, 257-261.

Ercole C., Vegliò F., Toro L., Ficara G. and Lepidi A. (1995) Immobilisation of microbial cells for metal adsorption and desorption. In: *Mineral processing II*, Holmes and Smith R.W. (Eds), 181-188.

Figueira M.M., Volesky B. and Ciminelli V.S.T. (1997) Assessment of interference in biosorption of a heavy metal, *Biotechnol. Bioeng.*, **54**, 344-350.

Fogel S. and Welch J.W. (1982) Tandem gene amplification mediates copper resistance in yeast, *Proc. Natl. Acad. Sci.*, **79**, 5342-5346.

Fourest E. and Roux J. (1992) Heavy metal biosorption by fungal mycelial by-products: Mechanisms and influence of pH, *Appl. Microbiol. Biotechnol.*, **37**, 399-403.

Fourest E., Canal C. and Roux J. (1994) Improvement of heavy metal biosorption by mycelial dead biomasses (*Rhizopus arrhizus*, *Mucor meihei* and *Penicillium chrysogenum*): pH control and cationic activation, *FEMS Microbiol. Rev.*, **14**, 325-332.

Friberg L., Kjellström T., Nordberg G. and Piscator M. (1979) Cadmium. In: *Handbook on the toxicology of metals*, Friberg L., Nordberg G. and Vauk V.B. (eds.), Elsevier/North-Holland Biomedical Press, Amsterdam.

Fuhrmann G. and Rothstein A. (1968) The transport of Zn^{2+}, Co^{2+} and Ni^{2+} into yeast cells, *Biochim. Biophys. Acta*, **163**, 325-330.

Gadd G.M. and Griffiths A.J. (1978) Microorganisms and heavy metal toxicity, *Microb. Ecol.*, **4**, 303-317.

Gadd G.M. and Mowell J.L. (1983) The relationship between cadmium uptake, potassium release and viability in *Saccharomyces cerevisiae*, *FEMS Microbiol. Lett.*, **16**, 45-48.

Gadd G.M. (1986) Fungal responses towards heavy metals, In: *Microbes in extreme environments*, Herbet R.A. and Codd G.A. (Eds), Academic Press Inc., Ltd., London, 84-110.

Gadd G.M. (1989) Green means clean: biomass and metal accumulation, *TIBTECH*, **7**, 325-326.

Gadd G.M. (1990) Heavy metal accumulation by bacteria and other microorganisms, *Experientia*, **46**, 834-840.

Gadd G.M. and Laurence O.S. (1996) Demonstration of high affinity Mn^{2+} uptake in *Saccharomyces cerevisiae*: Specificity and kinetics, *Microbiol.*, **142**, 1159-1167.

Gale N.L. (1986) The role of algae and other micoorganisms in metal detoxification and environmental clean-up, *Biotechnol Bioeng Symp*, **16**, 171-179.

Galun M., Keller P., Feldstein H., Galun E., Siegel S. and Siegel B. (1983) Recovery of uranium (VI) from solution using fungi II. Release from uranium-loaded *Penicillium* biomass, *Water, Air, and Soil Pollution*, **20**, 277-285.

Galun M., Keller P., Malki D., Feldstein H., Galun E., Siegel S. and Siegel B. (1984) Removal of uranium(VI) from solution by fungal biomass: inhibition by iron, *Water, Air, and Soil Pollution*, **21**, 411-414.

Gardea-Torresdey J.L., Tiemann K.J., Gonzalez J.H., Henning J.A. and Towsend M.S. (1996) Ability of silica-immobilized *Medicago sativa* (alfalfa) to remove copper ions from solution, *J. Hazardous Materials*, **48**, 181-190.

Garnham G., Codd G.A. and Gadd G.M. (1992a) Accumulation of cobalt, zinc and manganese by the estuarine green microalga *Chlorella salina* immobilised in alginate microbeads, *Environ. Sci. Technol.*, **26**, 1764-1770.

Garnham G.W., Codd G.A. and Gadd G.M. (1992b) Kinetics of uptake and intracellular location of cobalt, manganese and zinc in the estuarine green alga *Chlorella salina*, *Appl. Microbiol. Biotechnol.*, **37**, 270-276.

Garnham G.W., Codd G.A. and Gadd G.M. (1992c) Uptake of technetium by freshwater green microalgae, *Appl. Microbiol. Biotechnol.*, **37**, 679-684.

Gelmi M., Apostoli P., Cabibbo E., Porru S., Alessio L. and Turano A. (1994) Resistance to cadmium salts and metal absorption by different microbial species, *Current Microbiol.*, **29**, 335-341.

Giacasso P. (1996) The challenges of water supply, *Proc. biennial conference of the Water Institute of Southern Africa*, **1**, paper 1, Port Elizabeth.

Golab Z., Orlowska and Smith R.W. (1991) Biosorption of lead and uranium by *Streptomyces sp*, *Water, Air, and Soil Pollution*, **60**, 99-106.

Guibal E., Roulph C. and Le Cloirec P. (1992) Uranium biosorption by a filamentous *Mucor meihei* pH effect on mechanisms and performances of uptake, *Wat. Res.*, **26**, 1139-1145.

Hammack R.W. and Edenborn H.M. (1992) The removal of nickel from mine waters using bacterial sulphate reduction, *Appl. Microbiol. Biotechnol.*, **37**, 674-678.

Han N.S., Seo J. and Chung Y.C. (1992) Growth and copper resistance of recombinant *S. cerevisiae* containing a metallothionein gene, *Biotechnol. Lett.*, **14**, 7-11.

Hao Y., Roach A.L. and Ramelow G.J. (1993) Uptake of metals by non-living biomass derived from *Sphagnum* moss and water hyacinth roots, *J. Environ. Sci. Health*, **A28**, 2333-2343.

Harris P.O. and Ramelow G.J. (1990) Binding of metal ions by particulate biomass derived from *Chorella vulgaris* and *Scenedesmus quadricauda*, *Environ. Sci. Technol.*, **24**, 220-228.

Hart B.A. and Scaife B.D. (1977) Toxicity and bioaccumulation of cadmium in *Chlorella pyrenoidosa*, *Environ. Res.*, **14**, 401-413.

Holan Z.R. and Volesky B. (1994) Biosorption of lead and nickel by biomass of marine algae, *Biotechnol. Bioeng.*, **43**, 1001-1009.

Holan Z.R., Volesky B. and Prasetyo I. (1993) Biosorption of cadmium by biomass of marine algae, *Biotechnol. Bioeng.*, **41**, 819-825.

Hough J.S., Briggs D.E. and Stevens R. (1971) In: *Malting and brewing science*, Chapman and Hall Ltd., London.

Hough J.S. (1984) In: *The biotechnology of malting and brewing*, Cambridge University Press, Cambridge.

Huang C.P., Westman D., Quirk K. and Huang J.P. (1988) The removal of cadmium (II) from dilute aqueous solutions by fungal adsorbent, *Wat. Sci. Tech.*, **20**, 369-376.

Hutchins S.R., Davidson M.S., Brierley J.A. and Brierley C.L. (1986) Microorganisms in reclamation of metals, *Ann. Rev. Microbiol.*, **40**, 311-336.

Imahara H., Wakatsuki T., Kitamura T. and Tanaka H. (1978) Effect of copper on the growth of yeast, *Agric. Biol. Chem.*, **42**, 1173-1179.

Jensen T.E., Rachlin J.W., Jani V. and Warkentine B. (1982) An X-ray energy dispersive study of cellular compartmentalization of lead and zinc in *Chlorella Saccharophila* (Chlorophyta), *Navicula incerta* and *Nitzschia* (Bacillariophyta), *Environ. Experim. Botany*, **22**, 319-328.

Jirků V. (1995) Covalent immobilization as a stimulus of cell wall composition changes, *Experientia*, **51**, 569-571.

Joho M., Inouhe M., Tohoyama H. and Murayama T. (1995) Nickel resistance mechanisms in yeast and other fungi, *J. Ind. Microbiol.*, **14**, 164-168.

Jones R.P. and Greenfield P.F. (1984) A review of yeast ionic nutrition, *Process Biochem.*, **19**, 48-60.

Jones R.P. and Gadd G.M. (1990) Ionic nutrition of yeast - physiological mechanisms involved and implications for biotechnology, *Enzyme Microb. Technol.*, **12**, 402-418.

Kamizono A., Nishizawa M., Teranishi Y., Murata K. and Kimura A. (1989) Identification of a gene conferring resistance to zinc and cadmium ions in the yeast *Saccharomyces cerevisiae*, *Mol. Gen. Genet.*, **219**, 161-167.

Kaplan D., Heimer Y.M., Abeliovich A. and Goldsbrough P.B. (1995) Cadmium toxicity and resistance in *Chlorella* sp., *Plant Science*, **109**, 129-137.

Kapoor A. and Viraraghavan T. (1995) Fungal biosorption - an alternative treatment option for heavy metal bearing wastewaters: A review, **53**, 195-206

Kempster P.I., Hattingh W.H.J. and van Vliet H.R. (1980) Summarized water quality criteria, Department of Environment Affairs (SA),Technical Report Nº TR108.

Kessels B.G.F., Belde P.J.M. and Borst-Pauwels G.W.F.H. (1985) Protection of *Saccharomyces cerevisiae* against Cd^{2+} toxicity by Ca^{2+}, *J. Gen. Microbiol.*, **131**, 2533-2537.

Khalid A.M., Ashfaq S.R., Bhatti T.M., Anwar M.A., Shemsi A.M. and Akhtar K. (1993) The uptake of microbially leached uranium by immobilized microbial biomass. In: *Proc. Intl. Biohydrometallurgy Symp., Biohydrometallurgical Technologies II*, Torma A.E., Apel M.L. and Brierley C.L. (Eds), USA, 299-307.

Kim Y.H., Yoo Y.J. and Lee H.Y. (1995) Characteristics of lead adsorption by *Undaria pinnatifida*, *Biotechnol. Lett.*, **17**, 345-350.

Kratochvil D., Fourest E. and Volesky B. (1995) Biosorption of copper *Sargassum fluitans* biomass in fixed-bed column, *Biotechnol. Lett.*, **17**, 777-782.

Kumar S., Sastry S. and Mohan M. (1992) Use of wild type and nickel resistant *Neurospora crassa* for removal of Ni^{2+} from aqueous medium, *Biotechnol. Lett.*, **14**, 1099-1202.

Kuyucak N. and Volesky B. (1988) Biosorbents for recovery of metals from industrial solutions, *Biotechnol. Lett.*, **10**, 137-142.

Kuyucak N. and Volesky B. (1989a) The mechanism of cobalt biosorption, *Biotechnol. Bioeng.*, **33**, 823-831.

Kuyucak N. and Volesky B. (1989b) Desorption of cobalt-laden algal biosorbent, *Biotechnol. Bioeng.*, **33**, 815-822.

Lamb A. and Tollefson E.L. (1973) Toxic effects of cupric, chromate and chromic ions on biological oxidation, *Water Res.*, **7**, 599-613.

Lesuisse E., Crichton R.R. and Labbe P. (1990) Iron-reductases in the yeast *Saccharomyces cerevisiae*, *Biochim. Biophys. Acta*, **1038**, 253-259.

Lewis D. and Kiff R.J. (1988) The removal of heavy metals from aqueous effluents by immobilized fungal biomass, *Environ. Technol. Lett.*, **9**, 991-998.

Lichko L.P., Okorokov L.A. and Kulaev I.S. (1980) Role of vacuolar ion pool in *Saccharomyces carlsbergensis*: Potassium efflux from vacuoles is coupled with manganese or magnesium influx, *J. Bacteriol.*, **144**, 666-671.

Lovely D.R. and Phillips E.J.P. (1992) Bioremediation of uranium contamination with enzymatic uranium reduction, *Environ. Sci. Technol.*, **26**, 2228-2234.

Lovley D.R. (1995) Bioremediation of organic metal contaminants with dissimilatory metal reduction, *J. Indust. Microbiol.*, **14**, 85-93.

Losi M.E., Amrhein C. and Frankenberger W.T. (1994) Biodegradation and bioremediation; Bioremediation of chromate-contaminated groundwater by reduction and precipitation in surface soils, *J. Environ. Qual.*, **23**, 1141-1150.

Lotter L.H. and Pitman A.R. (1996) New approaches to the provision of water and sanitation in a metropolitan area, *Proc. biennial conference of the Water Institute of Southern Africa*, **1**, paper 12, Port Elizabeth.

Lui S. and Suflita J.M. (1993) Ecology and evolution of microbial populations for bioremediation, *TIBTECH*, **11**, 344-352.

Lundgren D.G., Valkova-Valchanova M. and Reed R. (1986) Chemical reactions important in bioleaching and bioaccumulation, *Biotechnol. Bioeng. Symp.*, **16**, 7-22.

Matis K.A., Zouboulis A.I. and Hancock I.C. (1994) Biosorption and flotation for metal effluent treatment, *Icheme Environ. Biotechnol.*, 86-88.

Matis K.A., Zouboulis A.I., Grigoriadou A.A., Lazaridis N.K. and Ekateriniadou L.V. (1996) Metal biosorption - flotation: Application to cadmium removal, *Appl. Microbiol. Biotechnol.*, **45**, 569-573.

Mattuschka B. and Straube G. (1993) Biosorption of metals by a waste biomass, *J. Chem. Tech. Biotechnol.*, **58**, 57-63.

Mason S.J. and Joubert A.M. (1995) A note on the inter-annual rainfall variability and water demand in the Johannesburg region, *Water SA*, **21**, 269-270.

Mehra R.K. and Winge D.R. (1991) Metal ion resistance in fungi: Molecular mechanisms and their regulated expression, *J. Cell. Biochem.*, **45**, 30-40.

Meintjies E. and Whitehead J. (1996) The pong in the bell rings an alarm, *Proc. biennial conference of the Water Institute of Southern Africa*, **1**, paper 23, Port Elizabeth.

Melzoch K., Rychtera M. and Hábová V. (1994) Effect of immobilization upon the properties and behaviour of *Saccharomyces cerevisiae* cells, *J. Biotechnol.*, **32**, 59-65.

Mergeay M. (1991) Towards an understanding of the genetics of bacterial metal resistance, *TIBTECH*, **9**, 17-24.

Mergeay M. and Springael D. (1997) Conjugation-mediated gene transfer in bacterial strains to be used for bioremediation. In: *Methods in Biotechnology, vol 2: Bioremediation Protocols*, Sheehan D. (Ed.), Humana Press Inc., Totowa, NJ, 153-167.

Mochaba F., O'Conner-Cox E.S.C. and Axell B. (1996a) Effects of yeast quality on the accumulation and release of metals causing beer instability, *J. Am. Brew. Chem.*, **54**, 164-171.

Mochaba F., O'Conner-Cox E.S.C. and Axell B. (1996b) Metal ion concentration and release by a brewing yeast: Characterisation and implications, *J. Am. Brew. Chem.*, **54**, 155-163.

Moo-Young M. and Chisti Y. (1994) Bioreactor applications in waste treatment, *Resources, Conservation and Recycling*, **11**, 13-24.

Mowell J.L. and Gadd G.M. (1983) Zinc uptake and the toxicity in the yeasts *Sporobolomyces roseus* and *Saccharomyces cerevisiae*, *J. Gen. Microbiol.*, **129**, 3421-3425.

Muraleedharan T.R. and Venkobachar C. (1990) Mechanism of biosorption of copper (II) by *Ganoderma lucidum*, *Biotechnol. Bioeng.*, **35**, 320-325.

Nakajima A., Horikoshi T. and Sakaguchi T. (1981) Studies on the accumulation of heavy metal elements in biological systems, *European J. Appl. Microbiol. Biotechnol.*, **12**, 76-83.

Nakajima A., Horikoshi T. and Sakaguchi T. (1982) Recovery of uranium by immobilised microorganisms, *European J. Appl. Microbiol. Biotechnol.*, **16**, 88-91.

Nakajima A. and Sakaguchi T. (1986) Selective accumulation of heavy metals by microorganisms, *Appl. Microbiol. Biotechnol.*, **24**, 59-64.

Nakajima A. and Sakaguchi T. (1993) Accumulation of uranium by basidiomycetes, *Appl. Microbiol. Biotechnol.*, **38**, 574-578.

Norberg A. and Rydin S. (1984) Development of a continuous process for metal accumulation by *Zoogloea ramigera*, *Biotechnol. Bioeng.*, **26**, 265-268.

Norris P.R. and Kelly D.P. (1977) Accumulation of cadmium and cobalt by *Saccharomyces cerevisiae*, *J. Gen. Microbiol.*, **99**, 317-324.

Norris P.R. and Kelly D.P. (1979) Accumulation of metals by bacteria and yeasts, *Dev. Ind. Microbiol.*, **20**, 299-308.

Northcote D.H. and Horne R.W. (1952) The chemical composition and structure of the yeast cell wall, *Biochem. J.*, **51**, 232-236.

Nourbakhsh M., Sağ Y., Özer D., Aksu Z. , Kutsal T. and Cağlar A. (1994) A comparative study of various biosorbents for removal of chromium (VI) ions from industrial waste waters, *Process Biochem.*, **29**, 1-5.

Nriagu J.O. and Pacyna J.M. (1988) Quantitative assessment of worldwide contamination of air, water and soils by trace metals, *Nature*, **333**, 134-139.

Nui H., Xu X.S., Wang J.H. and Volesky B. (1993) Removal of lead from aqueous solutions by *Penicillium* biomass, *Biotechnol. Bioeng.*, **42**, 785-787.

O'Halloran J., Walsh A.R. and Fitzpatrick P.J. (1997) The determination of trace elements in biological and environmental samples using atomic absorption spectroscopy. In: *Methods in Biotechnology, vol 2: Bioremediation Protocols*, Sheehan D. (Ed.), Humana Press Inc., Totowa, NJ, 201-211.

Ohsumi Y., Kitamoto K. and Anraku Y. (1988) Changes induced in the permeability barrier of the yeast plasma membrane by cupric ion, *J. Bacteriol.*, **170**, 2676-2682.

Okorokov L.A., Lichko L.P. and Kulaev I.S. (1980) Vacuoles: Main compartments of potassium, magnesium, and phosphate ions in *Saccharomyces carlbergensis* cells, *J. Bacteriol.*, **144**, 661-665.

Okorokov L.A. (1985) Main mechanisms of ion transport and regulation of ion concentration in the yeast cytoplasm. In: *FEMS Symposium 23, Environmental regulation of microbial metabolism*, Kulacv, Dawes and Tempest (Eds), Academic Press, London, 339-349.

Osborn D.W. (1989) Corrosion in potable water systems: The Johannesburg experience, *Water SA*, **15**, 201-208.

Pascucci P.R. (1993) Simultaneous multielement study of the binding of metals in solution by an algal biomass *Chlorella vulgaris*, *Analytical Lett.*, **26**, 445-455.

Palmer T. (1991) Understanding enzymes, Wiseman A. (ed.), Ellis Horwood Limited, Chichester, England.

Phaff H.J. (1971) Structure and biosynthesis of the yeast cell envelope. In: *The Yeasts, Physiology and biochemistry of yeasts*, vol 2, Rose A.H. and Harrison J.S. (eds), Academic Press, London, 135-210.

Pieterse M.J. (1989) Drinking water quality criteria with special reference to the South African experience, *Water SA*, **15**, 169-178.

Piscator M. (1979) Copper. In: *Handbook on the toxicology of metals*, Friberg L., Nordberg G. and Vauk V.B. (eds.), Elsevier/North-Holland Biomedical Press, Amsterdam.

Postek M.T., Howard K.S., Johnson A.H. and M[c]Michael K. (1980) In: *Scanning Electron microscopy: A students handbook*, Ladd Research Industries, Inc., 69-110.

Pümpel T. and Schinner F. (1993) Native fungal pellets as a biosorbent for heavy metals, *FEMS Microbiol Rev.*, **11**, 159-164.

Pümpel T., Pernfuß B., Pigher B., Diels L. and Schinner F. (1995) A rapid screening method for the isolation of metal-accumulating microorganisms, *J. Ind. Microbiol.*, **14**, 213-217.

Raguzzi F., Lesuisse E. and Crichton R.R. (1988) Iron storage in *Saccharomyces cerevisiae*, *FEBS Lett.*, **231**, 253-258.

Ramelow G.J., Fralick D. and Zhoa Y. (1992) Factors affecting the uptake of metal ions by dried seaweed biomass, *Microbios*, **72**, 81-93.

Ramelow U.S., Guidry C.N. and Fisk S.D. (1996) A kinetic study of metal ion binding by biomass immobilized in polymers, *J. Hazardous Materials*, **46**, 37-55.

Rose A.H. (1993) Composition of the envelope layers of *Saccharomyces cerevisiae* in relation to flocculation and ethanol tolerance, *J. Appl. Bacteriol. Symp. Supplement*, **74**, 110S-118S.

Rösick E., Mangir M. and Lochmann E.R. (1986) Unterschiedliche aufnahme von cadmium in *saccharomyces-bzw. Rhodotorula-zelen*, *Chemosphere*, **15**, 981-983.

Roy D., Greenlaw P.N. and Shane B.S. (1993) Adsorption of heavy metals by green algae and ground rice hulls, *J. Environ. Sci. Health*, **A28**, 37-50.

Sağ Y., Özer D. and Kutsal T. (1995) A comparative study of the biosorption of lead II ions to *Z. ramigera* and *R. arrhizus*, *Process Biochem.*, **30**, 169-174.

Sağ Y. and Kutsal T. (1996a) The selective biosorption of chromium (VI) and copper (II) ions from binary metal mixtures by *R. arrhizus*, *Process Biochem.*, **31**, 561-572.

Sağ Y. and Kutsal T. (1996b) Fully competitive biosorption of chromium (VI) and iron (III) ions from binary metal mixtures by *R. arrhizus*: Use of the competitive Langmuir model, *Process Biochem.*, **31**, 573-585.

Schubauer-Berigan M.K., Amato J.R., Ankley G.T., Baker S.E., Burkhard L.P., Dierkes J.R., Jenson J.J., Lukasewycz M.T. and Norberg-King T.J. (1993) The behaviour and identification of toxic metals in complex mixtures: Examples from effluent and sediment pore water toxicity identification, *Arch. Environ. Contam. Toxicol.*, **24**, 298-306.

Scott J.A. and Palmer S.J. (1990) Sites of cadmium uptake in bacteria used for biosorption, *Appl. Microbiol. Biotechnol.*, **33**, 221-225.

Sharma D.C. and Forster C.F. (1993) Removal of hexavalent chromium using sphagnum moss peat, *Wat. Res.*, **7**, 1201-1208

Sharma D.C. and Forster C.F. (1995) Continuous adsorption and desorption of chromium ions by sphagnum moss peat, *Process Biochem.*, **30**, 293-298.

Sharma D.C. and Forster C.F. (1995a) Column studies into the adsorption of chromium (VI) using sphagnum moss peat, *Biosource Technol.*, **52**, 261-267.

Shumate II S.E., Strandberg G.W., McWhirter D.A., Parrott J.R., Bogacki G.M. and Locke B.R. (1980), Separation of heavy metals from aqueous solutions using 'biosorbents' - development of contacting devices for uranium removal, *Biotechnol. Bioeng. Symp.*, **10**, 27-34.

Shuttleworth K.L. and Unz R.F. (1993) Sorption of heavy metals to the filamentous bacterium *Thiothrix* strain A1, *Appl. Environ. Microbiol.*, **59**, 1274-1282.

Simmons P., Tobin J.M. and Singleton I. (1995) Considerations on the use of commercialavailable yeast biomass for the treatment of metal-containing effluents, *J. Indust. Microbiol.*, **14**, 240-246.

Singh S.P., Singh R.K., Pandey P.K. and Pant A. (1992) Factors regulating copper uptake in free and immobilized cyanobacterium, *Folia Microbiol.*, **37**, 315-320.

Singleton I. and Simmons P. (1996) Factors affecting silver biosorption by an industrial strain of *Saccharomyces cerevisiae*, *J. Chem. Tech. Biotechnol.*, **65**, 21-28.

Slawson R.M., Trevors J.T. and Lee H. (1992) Silver accumulation and resistance in *Pseudomonas stutzeri*, *Arch. Microbiol.*, **158**, 398-404.

Sprey B. and Bochem H.P. (1981) Uptake of uranium into the alga *Dunaliella* detected by EDAX and LAMMA, *Fresenius Z. Anal. Chem.*, **308**, 239-245.

Stewart G.G. and Russell I. (1985) The biology of *Saccharomyces*. In: *Biology of industrial microorganisms*, Demain A.L. and Solomon N.A. (Eds), The Benjamin/Cummings Publishing Company, Inc., Menlo Park, California.

Stoll A. and Duncan J.R. (1997) Implementation of a continuous-flow stirred bioreactor system in the bioremediation of heavy metals from industrial wastewater, *Environ. Poll.*, In Press.

Strandberg G.W., Shumate II S.E. and Parrott J.R. (1981) Microbial cells as biosorbents for heavy metals: Accumulation of uranium by *Saccharomyces cerevisiae* and *Pseudomonas aeruginosa*, *Appl. Environ. Microbiol.*, **41**, 237-245.

Stephenson D. (1995) Factors affecting the cost of water supply to Gauteng, *Water SA*, **21**, 275-280.

Subramanian V.V., Sivasusubramanian V. and Gowrinathan K.P. (1994) Uptake and recovery of heavy metals by immobilized cells of *Aphanocapsa pulchra* (Kütz.) Rabenh., *J. Environ. Sci. Health*, **A29**, 1723-1733.

Sumino T., Nakamura H., Mori N. and Kawaguchi Y. (1992) Immobilization of nitrifying bacteria by polyethylene glycol prepolymer, *J. Ferment. Bioeng.*, **73**, 37-42.

Summers A.O. (1992) The hard stuff: Metals in bioremediation, *Current Opinion in Biotechnol.*, **3**, 271-276.

Talbot M.M.J., Branch E.B., Marsland S.A. and Watling R.J. (1985) Metal surveys in South African estuaries X. Blind, Ihlanza, Nahoon and Quinera rivers, *Water SA*, **11**, 65-68.

Tanaka H., Irie S. and Ochi H. (1989) A novel immobilization method for prevention of cell leakage from the gel matrix, *J. Ferment. Bioeng.*, **68**, 216-219.

Ting Y.P., Lawson F. and Prince I.G. (1991) Uptake of cadmium and zinc by the alga *Chlorella vulgaris*: II Multi-ion situation, *Biotechnol. Bioeng.*, **37**, 445-455.

Tobin J.M., Cooper D.G. and Neufeld R.J. (1984) Uptake of metal ions by *Rhizopus arrhizus* biomass, *Appl. Environ. Microbiol.*, **47**, 821-824.

Tobin J.M., Cooper D.G. and Neufeld R.J. (1988) The effects of cation competition on metal adsorption by *Rhizopus Arrhizus*, *Biotechnol. Bioeng.*, **31**, 282-286.

Tobin J.M., Cooper D.G. and Neufeld R.J. (1990) Investigation of the mechanism of metal uptake by denatured *Rhizopus arrhizus* biomass, *Enzyme Microb. Technol.*, **12**, 591-595.

Tobin J.M., L'homme B. and Roux J.C. (1993) Immobilisation protocols and effects of cadmium uptake by *Rhizopus arrhizus* biosorbents, *Biotechnol. Tech.*, **7**, 739-744.

Tobin J.M., White C. and Gadd G.M. (1994) Metal accumulation by fungi: Applications in environmental biotechnology, *J. Indust. Microbiol.*, **13**, 126-130.

Tohoyama H., Tomoyasu T., Inouhe M., Joho M. and Murayama T. (1992) The gene for cadmium metallothionein from a cadmium-resistant yeast appears to be identical to CUP1 in a copper-resistant strain, *Curr. Genet.*, **21**, 275-280.

Trevors J.T., Stratton G.W. and Gadd G.M. (1986) Cadmium transport, resistance, and toxicity in bacteria, algae, and fungi, *Can. J. Microbiol.*, **32**, 447-464.

Treen-Sears M.E., Volesky B. and Neufeld R.J. (1984) Ion exchange/complexation of the uranyl ion by *Rhizopus* biosorbent, *Biotechnol. Bioeng.*, **26**, 1323-1329.

Tsezos M. and Volesky B. (1981) Biosorption of uranium and thorium, *Biotechnol. Bioeng.*, **23**, 583-604.

Tsezos M. and Volesky B. (1982a) The mechanism of uranium biosorption by *Rhizopus arrhizus*, *Biotechnol. Bioeng.*, **24**, 385-401.

Tsezos M. and Volesky B. (1982b) The mechanism of thorium biosorption by *Rhizopus arrhizus*, *Biotechnol. Bioeng.*, **24**, 955-969.

Tsezos M. (1984) Recovery of uranium from biological adsorbents-desorption equilibrium, *Biotechnol. Bioeng.*, **26**, 973-981.

Tsezos M., Noh S.H. and Baird M.H.I. (1988) A batch reactor mass transfer kinetic model for immobilized biomass biosorption, *Biotechnol. Bioeng.*, **32**, 545-553.

Tsezos M., McCready R.G.L. and Bell J.P. (1989) The continuous recovery of uranium from biological leached solutions using immobilised biomass, *Biotechnol. Bioeng.*, **34**, 10-17.

Tsezos M. and Deutschmann A.A. (1990) An investigation of engineering parameters for the use of immobilized biomass particles in biosorption, *J. Chem. Tech. Biotechnol.*, **48**, 29-39.

Tsezos M., Georgousis Z. and Remoudaki E. (1997) Mechanism of aluminum interferance on uranium biosorption by *Rhizopus arrhizus*, *Biotechnol. Bioeng.*, **55**, 16-27.

Unz R.F. and Dietz J.M. (1986) Biological applications in the treatment of acidic mine drainages, *Biotechnol. Bioeng. Symp.*, **16**, 163-170.

van der Merwe W. and Grobler D.C. (1996) Water quality management in the RSA: Preparing for the future, *Water SA*, **16**, 49-53.

van der Rest M.E., Kammainga A.H., Nakano A., Anraku Y., Poolman B. and Konings W.N. (1995) The plasma membrane of *Saccharomyces cerevisiae*: Structure, function and biogenesis, *Microbiol. Rev.*, **59**, 304-322.

Velea I., Voicu A. and Lazar I .(1995) Biosorption of some metallic ions from industrial effluents using fungal strains and bacterial exopolysaccharides. In: *Biohydrometallurgical processing*, Jerez C.A., Vargas T., Toledo H. and Wiertz J.V. (Eds), 229-235.

Venkatasubramanian K. and Vieth W.R. (1979) Immobilized microbial cells. In: *Progress in Industrial microbiology*, vol. 15, Bull M.J. (Ed), Elsevier Scientific Publishing Company, New York.

Volesky B. (1987) Biosorbents for metal recovery, *TIBTECH*, **5**, 96-101.

Volesky B. (1992) Removal of heavy metals by biosorption, *Am. Chem. Soc.*, 462-466.

Volesky B., May H. and Holan Z.R. (1992) Cadmium biosorption by *Saccharomyces cerevisiae*, *Biotechnol. Bioeng.*, **41**, 826-829.

Volesky B. and Prasetyo I. (1994) Cadmium removal in a biosorption column, *Biotechnol. Bioeng.*, **43**, 1010-1015.

Volesky B. and May-Phillips H.A. (1995) Biosorption of heavy metals by *Saccharomyces cerevisiae*, *Appl. Microbiol. Biotechnol.*, **42**, 797-806.

Volesky B. and Holan Z.R. (1995) Biosorption of heavy metals, *Biotechnol. Prog.*, **11**, 235-250.

Vymazal J. (1987) Zn uptake of *Cladophora glomerata*, *Hydrobiolgia*, **148**, 97-101.

Wakatsuki T. (1995) Metal oxidoreduction by microbial cells, *J. Indust. Microbiol.*, **14**, 169-177.

Wang Y. and Shen H. (1995) Bacterial reduction of hexavalent chromium, *J. Indust. Microbiol.*, **14**, 159-163.

Watling R.J., Talbot M.M.J., Branch E.B. and Marsland S.A. (1985) Metal surveys in South African estuaries IX. Buffalo River, *Water SA*, **11**, 61-64.

Wehrheim B. and Wettern M. (1994) Biosorption of cadmium, copper, and lead by isolated mother cell walls and whole cells of *Chlorella fusca*, *Appl. Microbiol. Biotechnol.*, **41**, 725-728.

Werner-Washburne M., Braun E., Johnston G.C. and Singer R.A. (1993) Stationary phase in the yeast *Saccharomyces cerevisiae*, *Microbiol. reviews*, **57**, 383-401.

Westrin B.A. and Axelsson A. (1991) Diffussion in gels containing immobilized cells: A critical review, *Biotechnol. Bioeng.*, **37**, 439-446.

White C. and Gadd G.M. (1986) Uptake and cellular distribution of copper, cobalt and cadmium in strains of *Saccharomyces cerevisiae* cultured on elevated concentrations of these metals, *FEMS Microbiol. Ecol.*, **38**, 277-283.

White C. and Gadd G.M. (1990) Biosorption of radionuclides by fungal biomass, *J. Chem. Tech. Biotechnol.*, **49**, 331-343.

White C. and Gadd G.M. (1995) Determination of metals and metal fluxes in algae and fungi, *The Science of the Total Environment*, **176**, 107-115.

White C., Wilkinson S.C. and Gadd G.M. (1995) The role of microorganisms in biosorption of toxic metals and radionuclides, *Int. Biodeterioration and Biodegradation*, **35**, 17-40.

Wilde E.W. and Benemann J.R. (1993) Bioremoval of heavy metals by the use of microalgae, *Biotech. Adv.*, **11**, 781-812.

Winge D.R., Nielson K.B., Gray W.R. and Hamer D.H. (1985) Yeast metallothionein, *J. Biol. Chem.*, **260**, 14464-14470.

Wong P.K. and So C.M. (1993) Copper accumulation by a strain of *Pseudomonas putida*, *Microbios*, **73**, 113-121.

Wood J.M. and Wang H. (1983) Microbial resistance to heavy metals, *Environ. Sci. Technol.*, **17**, 582-590.

Yazgan A. and Özcengiz G. (1994) Subcellular distribution of accumulated heavy metals in *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*, *Biotechnol. Lett.*, **16**, 871-874.