Propagation and Characterization of *Rhizopus* Biosorbents

by

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A thesis submitted to the

Faculty of Graduate Studies and Research

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

June, 1986

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ISBN 0-315-38177-9

Abstract

Effects of growth conditions on biomass production and subsequent metal uptake behaviour of Rhizopus javanicus biosorbents (cell walls) have been examined. In defined glucose/urea/mineral salts media the biomass sequestered essential trace elements to the extent that growth was limited. Studies of mineral toxicities led to a high-salts medium which allowed rapid growth of R. javanicus to a concentration of 8 g biosorbent/L.

The biomass was grown at different rates in similar media by modification of pH, illumination, or timing of harvesting. Copper uptake by the biosorbent (modelled as ion exchange by one type of metal uptake site) was affected; the strength of binding decreased and the number of sites increased as the growth rate increased. Variations in uptake behaviour were related to changes in the cell wall structure during maturation.

The chemical composition of R. javanicus biosorbents was affected by the divalent cation content of the growth medium. These ions are known to act as cofactors or inhibitors of enzymes for synthesis of cell wall components (chitin/chitosan and poly-glucuronic acid). In turn, uptake of Cu(II), Mn(II) and Cr(III), which was chiefly due to ion exchange or complexation with phosphate and carboxyl groups, was affected by the biosorbent composition. Removal of ionic amine groups resulted in increased strengths of binding for all metals because the cations were then not electrostatically repelled.

Resumé

Les effets des conditions de la culture sur la production de la biomasse et sur l'insolubilisation ultérieure des metaux par biosorbents (parois des cellules) de Rhizopus javanicus ont été examinés. Dans les milieux de culture contenant glucose, urée, et des sels minéraux, la moisissure a absorbé des metaux essentiels, à tel point que la croissance fut limitée. Etudes sur la toxicité des mineraux ont résulté à un milieu contenant une grosse partie des sels, dans lequel R. javanicus a poussé rapidement à une concentration de 8 g biosorbent/L.

La croissance de la biomasse a été suivie à vitesses differentes dans des milieux de culture similaires, en modifiant soit le pH, l'éclairage, ou la date de la récolte. L'insolubilisation du cuivre par les biosorbents, mathematiquement modelé comme étant un échange d'ions sur un seul type de site, a été affectée: la force de la liaison a diminué et le nombre des sites d'adsorption a augmenté quand la vitesse de la croissance augmentait. Les variations quant à l'insolubilisation du metal ont été reliées aux changements qui ont pris place dans les parois des cellules pendant la maturation.

La composition chimique des biosorbents de R. javanicus a été affectée par les cations divalents du milieu de culture. Ces ions ont des rôles comme cofacteurs ou inhibiteurs des enzymes responsable de la synthèse des composants des parois cellulaires (chitine/chitosane et acide poly-glucuronique). En retour, l'insolubilisation du Cu(II), du Mn(II), et du Cr(III) a été affectée par la composition du biosorbent. L'insolubilisation a

été principalement due à l'échange des ions ou à la complexation par des groupes phosphates et carboxyliques. Les interactions électrostatiques avec les groupes amines cationiques ont diminué la force de la liaison avec tous les ions metalliques.

Acknowledgements

This work was carried out with the support of a Natural Sciences and Engineering Research Council Postgraduate Scholarship, in the Ottawa laboratories of the Division of Biological Sciences of the National Research Council. I would like to express my sincere gratitude to Dr. S.M. Martin who cheerfully inspired, criticized and facilitated all stages of this work, to Dr. C. Bishop for my support as a guest-worker, and to the employees of the N.R.C. for their assistance in innumerable ways.

I am grateful to my supervisor at McGill University, Dr. B. Volesky, as well as to Dr. R.J. Neufeld for encouragement of this work, and thoughts and criticisms. I have benefited from cooperation and stimulating discussions with many others, including Victoria Laube, who also read and commented on this manuscript, Dr. I.J. Macdonald and Dr. I. Veliky of N.R.C., Dr. V. Sanmugasunderam of Energy, Mines and Resources, and John Tobin of McGill. As well, Dr. F. Michon gave me advice on the chemical analysis of the biosorbents, Dr. F. Welsh carried out the HPLC analyses, and Dr. P.L. Sears provided the non-linear program for fitting of the metal uptake results.

Last, but far from least, I must thank my family and friends for their unending encouragement. In particular I thank my husband, Paul, who supported me every step of the way, and also read and corrected this manuscript. I am also very grateful to Christiane Stieber who generously and lovingly cared for our daughter for many hours during the writing of this work.

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1. INTRODUCTION

Plentiful supplies of clean water have long been taken for granted in the industrialized areas of North America, but the end of that situation is in sight. Water is becoming an increasingly valuable commodity, of steadily declining quality. There are many reasons for this. Acid rain and agricultural chemicals decrease the pH and increase the mineral, pesticide and herbicide content of water over large areas, while chemical dumps, mines and industries are point-sources of pollution. Governments attempt to regulate effluent compositions, setting supposedly safe limits on effluents, but unfortunately, given the complexity of the biological systems involved, these decisions are somewhat arbitrary. As well, the immediate financial cost of pollution control devices is a major factor in negotiations between governments and industries. P. D. Anderson (1981) answers the question of whether environmental quality criteria for individual substances provide adequate protection with a resounding "no". He describes "multiple toxicity", which arises from coexisting pollutants whose respective levels are within the permissible, ie. "safe" range, as "one of contemporary society's most insidious as well as ubiquitious Medusas".

The sum of small changes in the environment has global implications. G. W. Woodwell (1981) said, "Despite man's success in turning nature to his own purposes, we still live as guests in a biosphere dominated by natural communities that operate according to a complex and poorly recognized set of laws that have their basis in evolutionary processes. ... Sudden changes in environment, whether they are physical, chemical or biotic, will bring sudden changes in the biotic system of that place." One must keep in mind that "sudden changes" on an evolutionary time scale may not be immediately recognizable, being of the order of years. This has further implications, as Whitfield (1982) explains. As ecosystems are simplified by man's unintentional extermination of some species, the systems' balances become more fragile and the altered ecosystems are less resilient in the face of further changes in the environment.

The immediate problem in our society is that intensive agriculture and industry, and expanding urban populations require large supplies of high quality water. When the fact that we are living in a closed system becomes a political and economic reality, it is inevitable that more stringent controls on effluents will be imposed. Recovery of toxic materials from increasingly dilute solutions will have to be carried out. There are many commercial methods of waste-water treatment, based on one of two general principles. The contaminant may be precipitated by evaporation of the solvent, or adjustment of pH or addition of a counter-ion which forms a highly-insoluble salt, and subsequently separated by settling or filtration. Alternatively, pollutants may be collected by a second phase

such as ion-exchange resins, adsorbents such as activated charcoal, organic liquids containing water-insoluble ligands, or electrochemical devices (Patterson, 1977). Biosorption, the sequestering of metals by materials of natural (biological) origin, falls within the second method of contaminant recovery.

1.1 The Phenomena of Biosorption and their Significance

1.1.1 Metal uptake in the environment

The fact that organisms sequester metals from their environment has long been recognized, and in fact led to the first recognition of many components of sea-water as they were detected in seaweeds before methods were available to measure them directly (Riley, 1965, pp 23-24). In more recent years the quantity of metal associated with a variety of marine organisms, particularily algae, has led to the study of them as biological indicators of possible pollution problems arising from industrial effluents (Haug et al., 1974; Trollop and Evans, 1976). Biological monitoring of heavy metal pollution is not restricted to aqueous environments, however. Martin and Coughtrey (1982) reviewed monitoring of airborne pollutants and geological events, as well as geochemical prospecting in the terrestrial environment.

Major environmental damage may be incurred when mining and metal processing industries dump large quantities of biologically potent and valuable (but not economically recoverable) metals into the waterways. Many industries in major population centers are subject to more stringent effluent guidelines than are industries in sparsely populated areas, although the surrounding ecosystems are often not very resilient. However, streams do

tend to be self-cleansing, and much of the toxic metals in the water will be deposited en route to population centres. For instance, purification of water by algal meander systems has been reported in the Missouri lead-belt (Hassett, 1979; Gale and Wixson, 1979). The removal of metals from water flowing through swamps or beds of plants, both in fresh and saline water, is not uncommon and often involves cooperation among macro-species, which do not themselves bind large quantities of metals, and microbial populations attached to the stems and leaves (Anon., 1975; Corpe, 1975; Schlais, 1980). Bacteria in this environment not only sequester metals within the biomass or extracellular materials, but also affect the destiny of minerals. Microbes may oxidize, reduce, methylate or demethylate metals, or oxidize sulphide and solubilize metals as acid is produced (reviewed by both Silver, 1984; Torma and Bosecker, 1982).

Metals which are both toxic to the environment and valuable may be recovered from dilute solution to the benefit of all involved. One example of such serendipity arose in Flin Flon, Manitoba, where both municipal sewage and mining and smelting waste-waters were fed to a series of small lakes and streams. Trace metals were removed as they were immobilized by algae and entrained to the bottom, and as they were precipitated as sulphides. (Sulphate dumped by the mining operation was reduced to sulphide in the lower anoxic zones.) The sediments may eventually be mined and the metals recovered (Jackson, 1978). However, it is important to recognize that metals deposited in the sediments by such processes are not removed from the ecosystem entirely; and the possibility exists that they may

reappear in solution should the environment change. Nevertheless, the formation of a variety of minerals has been attributed to prolonged geological compaction and heating of organically bound metals in sediments (Ferguson and Bubela, 1974; Beveridge, 1980).

1.1.2 Metal uptake by cultured organisms and their products

1.1.2.1 Metal uptake by miscellaneous materials of biological origin

Biosorption is an ubiquitous phenomena and therefore has ramifications in many areas. In the soils and waterways the binding of metals by an "organic fraction", which often is classified as fulvic or humic acids, may affect significantly the cycling and deposition of metals in the environment (Giesey and Briese, 1978; Ramamoorthy and Rust, 1978). The modelling of metal complexation by humic materials has been discussed by Perdue and Lytle (1983). Metal uptake by a wide variety of organic materials has been examined. Peat moss bound 200 mmol/kg of Ni, Cu, Cd, and Zn at metal concentrations of 10 mM and pH>6.7, and all metals except for Ni could be recovered at pH <1.5 (Gosset and Trancart, 1986). Even filter paper has been examined for its ability to bind copper (Frew and Pickering, 1970), and it was found that at low solution concentrations ion exchange was responsible for uptake, while adsorption was responsible for uptake at concentrations above 1 mM. Masri et al. (1974) measured uptake of metals by a variety of materials including a selection of bark samples, leaves, needles, peat moss, lignin, orange peel and chitin, and found that "Milorganite" (an activated sewage sludge) exhibited higher uptake of Ca, Ti, Cr, Cu, Zn, Sr, Zr, Pb, and Hg than the other

materials. Mercury uptake by all the materials was greater than uptake of the other cations. In another experiment chitosan took up more Hg, Cd, Pb, Zn, Co, Ni, Cu, Ag, Pt and Pd than the activated sludge, and it commonly exhibited an uptake capacity greater than 1000 μmol/g. However, no mention was made of the concentrations or pH of the metal solutions.

There has been considerable interest in metal uptake by chitin and chitosan. Chitin is a major component in shells and is available in large quantities as a waste from sea-food processing. Deacetylation of chitin (β-1→4, N-acetyl glucosamine) to form chitosan greatly enhances the uptake of many metals, particularily copper (Yang and Zall, 1984) and metal uptake by chitosan has been studied extensively. Uptake is due to complexation with the amine nitrogen and is decreased significantly below pH~6 (Yaku and Koshijima, 1978). The capacity of the polymer for copper may be enhanced by factors of 2.2-47 by preparing the carboxylmethyl derivative, or by reacting the amine group with salicylaldehyde and subsequently reducing the azomethine group with cyanoborohydride (Hall and Yalpani, 1980). One problem with the use of chitosan as a biosorbent is its solubility in acid, which may preclude metal recovery at low pH in industrial applications. Solubility problems may be circumvented by cross-linking with gluteraldehyde, but results in decreased metal uptake (Masri and Randall, 1978; Yang and Zall, 1984). Metal recovery from commercial chitosan by short-term exposure to sulphuric acid at concentrations less than 3% has been reported to result in little deterioration of the particles (Hauer, 1978). Chitosan, and a chitosan-glucan complex from

Aspergillus niger, have been used in columns for separation and collection of metallic ions (Muzzarelli *et al.*, 1969, 1970, 1980a,b; Muzzarelli and Sipos, 1971).

1.1.2.2 Metal uptake by algae

Algae (as discussed above) have been used as indicators of the extent of water contamination. Their analysis has also contributed to an understanding of the cleansing of industrial waste-waters in meander systems. Metal uptake by isolated algal cultures has also been studied. Sequestering by *Vaucheria* (Crist *et al.*, 1981) was ascribed to ion exchange on the surface (cell wall). A cation exchange capacity of 1000 μmol/g was measured by titration with NaOH between pH 3 and 8. Ferguson and Bubela (1974) examined metal sequestering by species of *Ulothrix* and *Chlamydomonas*, and *Chlorella vulgaris*. They postulated that ion exchange was the main mechanism of uptake because the data could be fitted to Langmuir's equation and sorption was strongly supressed at lower pH values. Data from low solution metal concentrations (<0.2 mM) could be extrapolated to an uptake capacity of about 500 μmol divalent cation/g biomass for two of the algae, but the *Chlorella* biomass in contact with higher metal concentrations exhibited anomalously high values of copper uptake. This was attributed to precipitation of copper phosphates. It was also noted that zinc was quite weakly bound as it could be displaced by sodium and magnesium. Mixed algal cultures collected from Missouri's lead belt exhibited cation exchange capacities up to 640 μeq/g biomass on the basis of lead uptake (Gale and Wixson, 1979). This uptake corresponded to the release of other cations associated with the

cell walls and was reversed by washing with EDTA. Large differences in the tolerance of living organisms to loading with lead and zinc were observed, and the significance of this in the removal of vagrant metals in contact with growing algae was noted.

Accumulation of cadmium by Chlorella vulgaris specimens grown in media with and without the metal has been studied, and it was found that uptake over an extended period of cultivation was greater than would be expected due simply to surface adsorption (Khummongkol et al., 1982). The extra accumulation was ascribed to intracellular deposition of the metal, even though no evidence of such localization was presented, and the possible effects of cadmium in the growth medium on the cell wall were not discussed. The observation that the metal uptake by older cells was less than that by younger cells was ascribed to changes in the medium composition, recognizing that medium components may complex the metal and thereby change the uptake behaviour. However, the possibility that the cells themselves might be different was not recognized.

1.1.2.3 Metal uptake by bacteria

Metal taken up by bacteria may be located in the cytoplasm, extracellular polysaccharides or cell wall.

Active transport into the cytoplasm, and immobilization of metals in vesicles or granules, are the results of relatively slow processes which are sensitive to both temperature and metabolic inhibitors (Lester,

1983). Such accumulation may be associated with mechanisms of resistance to toxic metals in the growth medium. Alternatively, increased specific transport of metals out of the cell may result in decreased accumulation by resistant species (Silver, 1984). Bollag and Duszota (1984) found no correlation between cadmium-resistance and uptake abilities of a variety of bacteria. Presumably individual species adopted one of these two diametrically opposed mechanisms of adaptation, involving either enhanced immobilization or exclusion of the toxic metal by the cells.

In many studies of bacterial metal uptake using whole cells, extracellular polysaccharides or capsular materials play a significant role. Lead recovery by non-capsulated Micrococcus luteus was about one-sixth of that by capsulated Azotobacter sp. when both were grown in media containing lead (Tornabene and Edwards, 1972). Similarly, high uptake of cadmium by growing and resting Citrobacter sp., compared to uptake by other species under investigation, was possibly due to an exopolymer (Macaskie and Dean, 1982). Capsulated Bacillus megaterium was found to bind non-toxic divalent cations, and to be less sensitive to copper, mercury and silver than a small-capsule mutant (Cassity and Kolodziej, 1984). Copper, uranium and cadmium bound by Zoogloea ramigera biomass (17% cells; 83% polysaccharide) was recovered in acid solution, and the biomass could be reused (Norberg and Persson, 1984). Emulsan, the isolated extracellular polymer of Acinetobacter RAG-1, effectively bound uranium. This was ascribed to complexation by uronic acid residues which make up about 33% of the polymer (Zosim et al., 1983). Strength of

binding was enhanced when the polymer was constrained by adsorption on a hexadecane-water interface. Metal accumulation by microbially produced polymers, with emphasis on subsequent floc formation to aid separation and recovery of metals and organic material, was reviewed by Dugan and Pickrum (1972).

Beveridge and Murray (1980) examined uptake of a variety of metals by Bacillus subtilis cell walls by chemically modifying ionizable groups. They demonstrated complexation/ion exchange and precipitation of hydroxides or salts within cell walls. The removal of teichoic acid (phosphate) resulted in stoichiometric reduction in uptake, while nucleation of microcrystals was associated with the carboxylate groups. Binding of a cationic osmium probe corresponded to the carboxylate groups of the peptidoglycan and was affected little in teichoic acid-depleted walls (Beveridge and Jack, 1982). Metal binding by Escherichia coli K-12 cell walls has also been attributed to the carboxyl groups of the peptido-glycan sacculus (Hoyle and Beveridge, 1983, 1984).

1.1.2.4 Metal uptake by fungi

Studies of metal uptake by some fungi initially arose from concerns regarding fungicides and the subsequent fermentability of juices, but investigations now focus on more basic topics. The pattern of copper uptake by Aureobasidium pullulans was found to depend upon the form of the organism (Gadd and Mowll, 1986). Yeast and mycelia exhibited initial rapid, metabolism-independent binding to the cell surface, followed by slower uptake which was metabolism-dependent. Chlamydospores exhibited much greater

uptake, all of which was metabolism-independent. Initial binding by the mycelium was reduced by 43% at pH 2.5 compared to pH 6.5, while uptake by the yeast-like cells and chlamydospores was reduced by 77% and 84% respectively. Similar biphasic uptake behaviour was observed for zinc accumulation by the yeasts Sporobolomyces roseus and Saccharomyces cerevisiae (Mowll and Gadd, 1983). In S. cerevisiae cadmium resistance was found to correspond to decreased uptake by the cell wall, lower levels of the metal in the cytoplasm, and the production of a low molecular weight metal-specific protein in the cytoplasm (Joho et al., 1985).

Studies of metal uptake by moulds has more frequently arisen from interest in biosorbents. Both growing and pre-cultured Penicillium biomass samples sequestered uranyl ion from solution (Zajic and Chiu, 1972; Galun et al., 1983a). The presence of uranium in the medium had a deleterious effect on metal uptake by the growing organism, while treatments to kill the mould did not decrease, and in some cases enhanced uptake. Significant uptake was observed at pH 2.5, and this was ascribed to binding by phosphate or sulphate groups. With a view to commercial exploitation of a biosorbent, investigations were carried out using pre-cultured whole cells, which revealed that iron (III) severely interfered with uranium uptake, although divalent Ni, Cu, Zn, Cd and Pb as well as molybdate and chromate had relatively little effect. Uranium could be efficiently recovered from the biomass using alkali carbonates; 61% recovery was obtained in 0.1 M EDTA and little was recovered in aqueous

ammonia or organic acid solutions at pH 4-6 (Galun et al., 1983b, 1984).

A series of studies of Rhizopus biosorbents was initiated when Tsezos and Volesky (1981) discovered that Rhizopus arrhizus was the best of a variety of waste biomass samples for sequestering uranium and thorium from solution. The metal was associated with the cell wall. and uptake was ascribed to coordination with chitin nitrogen, as well as precipitation and hydrolysis (Tsezos and Volesky, 1981, 1982a,b). Tobin et al. (1984) measured uptake of a variety of metal cations by R. arrhizus biomass and proposed that uptake was primarily due to association with phosphate and carboxyl groups, based on the fact that uptake was a function of the ionic radius of the metal ions. Treen-Sears et al. (1984) studied uptake of the uranyl ion by species of Rhizopus and found that although the ability to sequester uranium effectively was a characteristic of the genus, the growth conditions and medium composition significantly affected this ability. A model of ion exchange or complexation described uptake by a sample of R. oligosporus, in that uptake was found to be largely reversible upon adjustment of pH to a value less than pH 2, or addition of salts or ligands such as EDTA. Rhizopus arrhizus was propagated as small, strong pellets, which when packed in a column repeatedly collected uranium from dilute neutral solution and released it in a more concentrated form in 0.1-1 M acid solutions. Ion exchange of 2 H^+:1 UO_2^{2+} was observed during the course of this study.

There are a few reports of comparisons of metal uptake by a variety of organisms. In an investigation of cadmium uptake by eleven soil microorganisms, Streptomyces sp. and the mould Mucor racemosus exhibited the greatest ability to sequester the metal (Kurek et al., 1982). Cadmium uptake by a large number of organisms growing in dilute media was studied by Macaskie and Dean (1982), who determined that there was no significant difference in general between the metal uptake ability of bacteria and moulds. They studied no mucoralean fungi. In a rather poorly documented study of mercury uptake by unspecified yeasts and bacteria, Glombitza et al. (1984) found that bacteria cultivated on methanol sequestered more than twice as much metal as yeast cultivated on hydrocarbons. Horikoshi et al. (1981) examined sequestering of the uranyl ion by a variety of organisms and concluded that in general the order of affinity was actinomycetes > bacteria > yeasts > moulds, although there were exceptional species. Rhizopus oryzea was the only mould examined which effectively sequestered uranium. Tsezos and Volesky (1981) also examined uptake of the uranyl ion by several samples of waste biomass and found that Rhizopus arrhizus was the most effective biosorbent among those studied. It is of interest that in all of the studies in which mucoralean fungi were included among the species examined, these organisms were among the best for removal of both cadmium and uranium from solution. This provides some rational for examination of Rhizopus biosorbents in the present work.

1.1.3 Industrial applications of biosorbents

In populous areas the natural resiliency of the ecosystems cannot be depended upon to immobilize toxic metals dumped by many industries. Passive metal recovery systems such as the Flin Flon or Missouri examples are not universally applicable because intensely populated areas do not have large land areas free for such applications. As well, growth in waste waters is often inhibited by metal toxicity (Hatch and Menawat, 1978). Other methods of reduction of levels of toxic metals have been devised which involve collection of metals by microorganisms growing in the waste in biological reactors, such as collection of metals from metal-finishing wastes by bacteria used to reduce nitrate levels (Shumate et al., 1980; Hollo et al., 1980). Anaerobic digestion and sewage treatment of dilute metal-containing streams also may result in immobilization of substantial quantities of heavy metals from dilute streams (Callander and Barford, 1983a,b; reviewed by Lester, 1983). Although propagation of organisms in a smaller, contained biological reactor to remove undesirable metallic ions from wastes in a manner similar to the natural immobilization processes is elegant in concept, it is not widely applicable due to the toxicity of effluent streams. Mineral levels must be reduced by a pretreatment step or by dilution, or the toxic metals may destroy the population of a man-made reactor.

The fact that biosorption of metals is often attributable to the physical-chemical make-up of the biological material suggests that toxicity problems could be avoided by propagating biomass and subsequently using it to take up metal ions. In terms of

industrial processes, one could envisage either disposable biosorbents, or a reusable biological ion-exchange resin or 'sorbent. This approach was evident in the course of research discussed above, and was noted by Macaskie and Dean (1982).

Several applications of biosorbents are presently patented or marketed:

- Chitosans of various descriptions are commercially available, and have been compared favourably with other weak-base anion exchange or chelating resins (Hauer, 1978). Characterization of these materials has been discussed by Muzzarelli et al. (1981a).
- Derivatives of chitin, chitosan and other polysaccharides which are useful for complexing metals as well as other applications have been patented by Hall and Yalpani (1984).
- Algae, immobilized in silica or polyacrylamide gels, and capable of immobilizing 5-10 times as much metal as conventional ion exchange resins are being licensed by New Mexico State University (Anon., 1985). Preparations bind gold very strongly and are tolerant to acid and high salts concentrations.
- Selective 'sorbents prepared by immobilization of microbial siderophores (extracellular chelating molecules) have been patented by Devoe-Holbein Inc. (1983).
- Application of a proprietary microbially-derived metal recovery agent has been engineered and commercialized by Advanced Mineral Technologies (Golden Colorado, N.M., U.S.A.). The 'sorbent is amorphous, irregularily-shaped pellets which shrink as they take up metal. Counter-current fluidized bed continuous operation pilot plants are available (Anon., 1986).
- Rhizopus biosorbents have been patented by Volesky and Tsezos (1981).

1.2 Description, Taxonomy and Growth of Species of Rhizopus

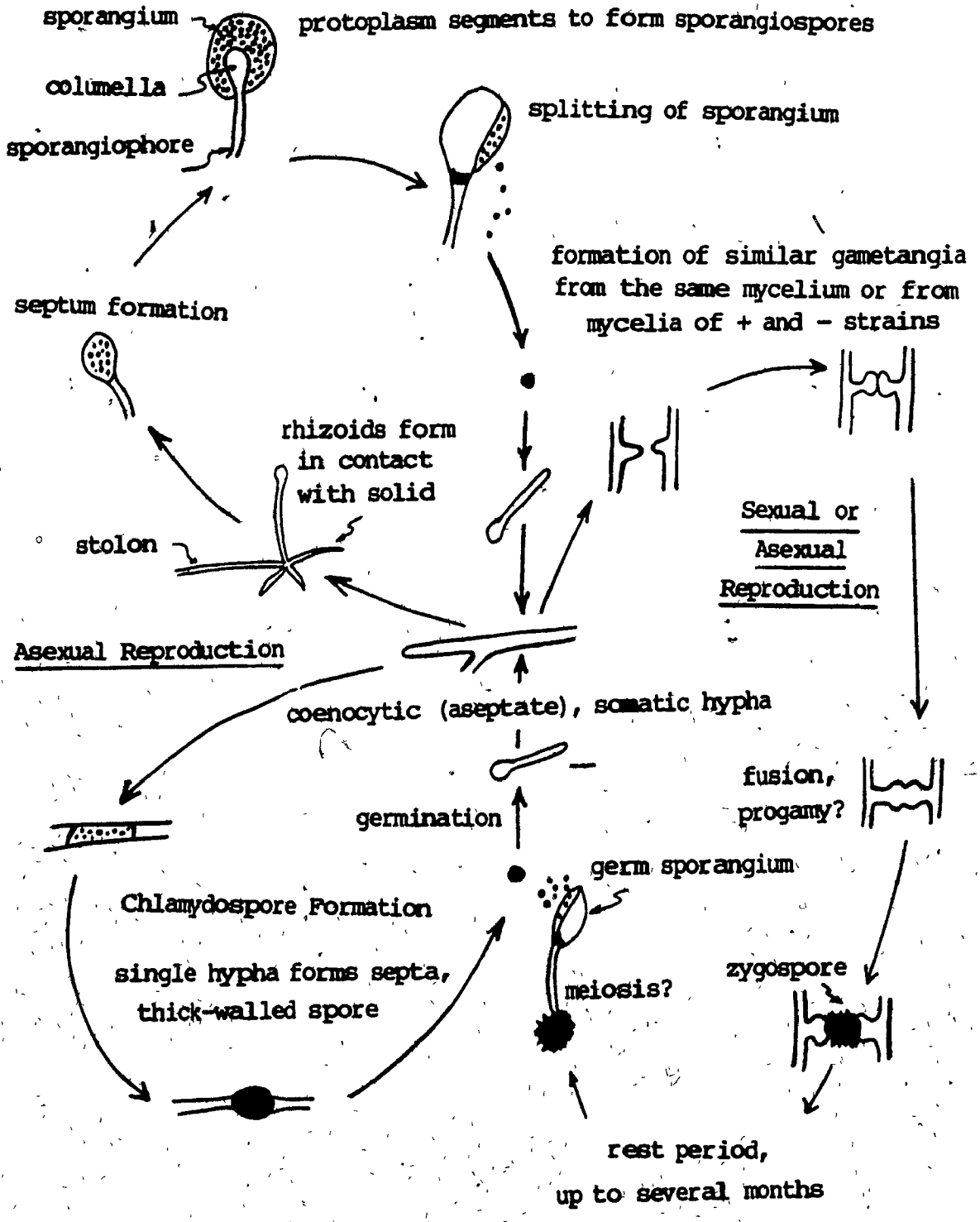
Rhizopus species are ubiquitous in the environment and cause rot of fruit, vegetables and other foods. Species capable of growth at 37°C may cause mucormycosis in mammals (Hesseltine and Ellis, 1973). Extracellular products of Rhizopus which are economically significant are organic acids, particularily lactic and fumaric (ibid), lipases, proteases, amylases and pectic enzymes. Some species also carry out selective transformations of steroids (Fogarty, 1983). Rhizopus species have long been used in the preparation of oriental foods as they are present in chinese yeast and starter cultures which break down starch and protein before fermenting beans or other materials for soya sauce or alcohol production, or for production of tempeh (moulded soya beans) (Inui et al., 1965; Hesseltine, 1985). Rhizopus oligosporus, which is a common organism for tempeh production, also produces heat-stable antibacterial compounds which aid in combating infections in those consuming it (Steinkraus et al., 1983).

Taxonomy of fungi is extensively debated, in part because little is known of their origin and evolution, but also because these rather complicated organisms may exhibit different morphology, metabolism, chemical composition and modes of reproduction when grown under different conditions. The genus Rhizopus is classified according to Alexopolous and Mims (1979, pp 37-39, ch. 9) in the division Amastigomycota (fungi with no centrioles but functioning spindle pole bodies; no motile cells), subdivision Zygomycotina (saprobic or parasitic fungi, typically coenocytic mycelium, asexual reproduction usually by

sporangiospores, sexual reproduction by fusion of gametangia to form zygosporangia containing zygospores), class Zygomycetes (sexual reproduction by fusion of usually equal gametangia ...), order Mucorales (mostly saprobic, coenocytic hyphae with septa principally at the base of reproductive structures), and family Mucoraceae (the largest and most primitive family in the order).

Figure 1.1 illustrates three life cycles of Rhizopus, although generally one or two cycles predominate for any particular species grown under given conditions. Rhizopus is a member of Zygomycetes in spite of the fact that few species have actually been observed to form zygospores. Rhizopus sexualis forms homothallic zygospores, while R. stolonifer and R. nigricans form heterothallic zygospores upon the mating of + and - strains.

Figure 1.1: Growth and Reproduction of Rhizopus



1.3 Objectives

This work is a continuation of a Master's thesis on Rhizopus biosorbents (Treen, 1981), the main results of which were published (Treen-Sears et al., 1984). Although the genus Rhizopus was found to exhibit very good biosorbent properties, it was observed that the uranium uptake ability of R. oligosporus was strongly affected by the growth conditions. The desire to propagate biosorbents with different metal uptake characteristics led to this detailed study of propagation of R. javanicus and characterization of its biosorbents. The three main objectives were to:

1. Establish methods for defined and reproducible production and assessment of biosorbents. This includes development of synthetic media in which to propagate large quantities of Rhizopus biosorbent.
2. Assess the effects of growth conditions on biosorbent production (both quantity and quality).
3. Study the differences in metal uptake behaviour and composition of biosorbents isolated from biomass propagated under different conditions which affected the biosorbent quality.

2. MATERIALS AND METHODS

All water used in the course of this work, for biosorbent preparation as well as all other experiments, was distilled and then deionized (Barnsted 4-cartridge water purification columns, Sybron; columns less than 2% exhausted), and all chemicals were analytical reagent grade, unless it is noted that they were purified further.

2.1 Propagation of Rhizopus and Preparation for Further Analysis

2.1.1 Growth of biomass

The Rhizopus species used in these studies are listed in Table 2.1.

All organisms were maintained on an organic medium (glucose, 20 g/L; neopeptone, 10 g/L; $NaNO_3$, 1 g/L; $MgSO_4$, 0.5 g/L; and agar, 20 g/L) to avoid loss of the organism should it not grow well on a synthetic medium. Synthetic media (Table 2.2) were prepared by steam-sterilizing a solution of glucose and most salts before mixing with filter-sterilized urea and KH_2PO_4 solutions. This minimized hydrolysis of the urea and caramelization of the glucose.

Table 2.1

Cultures of *Rhizopus*

	American Type Culture Collection No.	National Research Council Culture Collection No.
R. arrhizus Fisher	-- *	2828
R. chinensis Saito	22958	2834
R. delemar var. *multiplici-sporus* Inui *et al.*	24864	2829
R. formosaensis Nakazawa	26612	2835
R. japonicus Vuillemin	24863	2836
R. javanicus Takeda	22580	2830
R. oligosporus Saito	22959	2831
R. oryzea Went *et* Prinsen-Geerlings	12883	2832

* obtained from Canada Packers, Toronto, Canada

Table 2.2
Synthetic Media for Propagation of Rhizopus Species

	units*	Preliminary Media (A)	(A2)	(B)	Unsupplemented Medium (C)	High Salts Medium (HSM)	Screening Medium (SM)	Minimal Medium (MM)	High Ca, Zn Medium (HCZ)
glucose	(g/L)	30	10	3.3	1	20	20	10	20
urea	(g/L)	3	1	0.33	0.2	4	4	4	4
KH_2PO_4	(g/L)	3	1	0.33	0.4	4	4	3	4
K_2HPO_4	(g/L)	-	-	-	-	-	-	1.2	-
$MgSO_4$	(μM)	0.3 g/L	0.07 g/L	0.5 g/L	0.5	8000	8000	4	4000
$CaCl_2$	(μM)	0.2 mg/L	0.01 mg/L	0.07 mg/L	0.04	9000	40	4	18000
$FeCl_3$	(μM)	2 mg/L	2 mg/L	0.7 mg/L	4	400	40	4	400
$MnSO_4$	(μM)	20 μg/L	20 μg/L	7 μg/L	0.04	40	4	0.4	40
$ZnSO_4$	(μM)	40 μg/L	40 μg/L	14 μg/L	0.04	40	4	0.4	80
$CrK(SO_4)_2$	(μM)	20 μg/L	20 μg/L	7 μg/L	0.04	0.5	0.4	0.4	0.4
$(NH_4)_6Mo_7O_{24}$	(μM)	20 μg/L	20 μg/L	7 μg/L	0.04	0.5	0.4	0.4	0.4
$CoCl_2$	(μM)	40 μg/L	40 μg/L	14 μg/L	0.04	0.04	0.04	0.04	0.04
$NiCl_2$	(μM)	40 μg/L	40 μg/L	14 μg/L	0.04	0.04	0.04	0.04	0.04
$CuSO_4$	(μM)	2 μg/L	2 μg/L	0.7 μg/L	0.04	0.04	0.04	0.04	0.04

* unless otherwise stated

All cultures were incubated at 25°C. Cultures on solid media were grown within 1 m of a 40-W incandescent lamp for 5 to 7 days to achieve healthy sporulation. Stock cultures were then stored at 4°C with routine transfers every 3 months. Before spore suspensions were prepared as inocula for liquid media, the organism was subjected to a minimum of 5 transfers (a loop was used to transfer a small quantity of aerial growth) on agar (20 g/L) slants of the same medium. Spore inocula were prepared aseptically in two steps. Water (5 mL) was added to a freshly grown agar slant and agitated, and 1 mL of the spore suspension was used to inoculate a 30 cm^2 agar surface. After 5 to 7 days of growth, the spores were suspended in water, filtered through a 80-μm mesh nylon screen, and collected on a 0.45 μm membrane filter. The spores were rinsed and suspended in water before inoculation of the liquid medium. Spore concentrations were determined using a Petroff-Hauser counting chamber. Although spore inocula were used in the majority of experiments, a disperse vegetative inoculum was used in one experiment, as indicated. These were young (early-exponential phase) cultures.

The moulds were grown in suspension in Erlenmeyer flasks, Wheaton bottles and fermentors. The type of growth vessel is noted in each experiment. In general, shake flask experiments were carried out with 50 mL or 100 mL of medium in 250-mL Erlenmeyer flasks stoppered with foam plugs, and agitated at 250 rpm, 2.5 cm displacement on a rotary shaker. Sealed and agitated 150-mL Wheaton bottles containing 50 mL of medium were used in an investigation of dimorphic growth. All shake flask experiments were performed in duplicate or triplicate, although

greater numbers of flasks were used when larger quantities of biomass than would be produced in only a couple of flasks were required for metal uptake determinations. Water-jacketed, 2-L Versatec fermentors (Pegasus Industrial Specialities, Agincourt, Ontario, Canada) with 1.5 L of broth were also used. Dry air was metered, moistened, filtered through sterile glass wool, and supplied to the base of the agitator shaft below a 6.5-cm diameter stainless steel horizontal mixing plate. The plate had 21 holes with bevelled edges pointing down, so that when the shaft was vibrated vertically (60 Hz) by a "Vibra-Mixer" (Pegasus Ind. Special.) downward pumping action resulted. The pH was controlled by automatic addition of 2.0 M NaOH (Chemtrix, type 45). Samples were withdrawn through a tube, which was kept clear when not in use with a slow flow of air. Propagation of larger quantities of biomass for extraction and detailed analyses of biosorbent composition and behaviour was carried out in 10 L of medium at pH 6 in 14-L fermentors (Microferm, New Brunswick Scientific Co., NJ, USA). The cultures were aerated at 10 L/min and agitated at 400 rpm by two 4-blade turbine impellers of 10 cm diameter, with 2-cm square blades.

Glucose in the fermentation broth was measured using the DNS reducing sugar assay (Fischer and Kohtes, 1951). A 2-mL sample containing less than 2 mg of glucose was mixed with 2 mL of reagent (3,5-dinitrosalicylic acid, 10 g/L; sodium hydroxide, 16 g/L; and sodium potassium tartrate, 300 g/L) and heated for five minutes at 100°C. After cooling, 6 mL of water was added, the sample was mixed well and the absorbance was measured at 560 nm using round 1-cm glass test tubes in a Beckman spectrophotometer.

There was no interference from components of the growth media.

2.1.2 Harvesting and extraction of biomass

Biomass was routinely harvested by filtration on paper (Whatman medium). It was then resuspended in water in a Waring blender at high speed for 15-30 s, filtered, resuspended in 0.1 M HCl in the blender, filtered, and repeatedly resuspended in water in the blender and filtered until the clear, colourless filtrate was of neutral pH. The pale, paper-like biosorbent (material for metal uptake studies) was then either dried at room temperature if the samples were very small, or at 50°C in a vacuum oven. Microscopic examination revealed hyphal ghosts and debris. Very little, if any, of the cytoplasm remained after the acid treatment.

Three large batches of biomass for extraction and detailed analyses of composition and metal uptake behaviour, which were propagated in the Microferm fermentors, were repeatedly suspended in water in a Waring blender, dried and then ground to a fine powder in a ceramic ball mill. To prepare standard and extracted biosorbents 3-g quantities of powdered biomass were mixed continuously at room temperature in 500 mL of either 0.01 M HCl or 3 M HNO_2 for 3 h, or in 5 M LiCl overnight. The nitrous acid extraction was carried out by mixing equal quantities of cold $NaNO_2$ and HCl solutions with the biomass in a 5-L flask (some initial foaming occurred) and covering the mouth with plastic film (Parafilm) to minimize leakage of gas. The extracted biosorbents were centrifuged, and repeatedly washed with water until all water samples were colourless and of neutral pH.

2.2 Determination of Metal Uptake

The biosorbent was dried and broken up or ground, and then

Table 2.3

Wavelengths and Molar Extinction Coefficients for the Analysis of DEDTC-Metal Complexes

Species	Salt	Wavelength (nm)	Molar Extinction Coefficient (L/mol/cm)
Mn (II)	$MnSO_4 \cdot H_2O$	354	5.0×10^3
Fe (III)	$FeCl_3 \cdot 6H_2O$	347	7.0×10^3
Co (II)	$CoCl_2 \cdot 6H_2O$	325	26×10^3
Ni (II)	$NiCl_2 \cdot 6H_2O$	324	38×10^3
Cu (II)	$CuSO_4 \cdot 5H_2O$	433	13×10^3 *
Zn (II)	$ZnSO_4 \cdot 7H_2O$	262	17×10^3
Cd (II)	$Cd(NO_3)_2 \cdot 4H_2O$	262	20×10^3
Ag (I)	$AgNO_3$	262	9.6×10^3
Pb (II)	$Pb(NO_3)_2$	262	18×10^3
Hg (II)	$HgCl_2$	275	32×10^3
Cr (III)	$CrK(SO_4)_2 \cdot 12H_2O$	300	6.0×10^3

* For comparison, reported values for Cu II complexes are $12.1\text{-}13.4 \times 10^3$ (Hulanicki, 1967).

accurately weighed with an analytical balance (0.05 g to 0.1 g) for metal contacting experiments. Either 20 or 25 mL of metal-bearing solution was shaken with the biosorbent at 25°C for 15 to 20 h (experiments outlined in Section 3.1.3 showed that equilibrium was reached after 10 h), the pH was measured, and samples were examined microscopically for evidence of microbial growth.

Most of the metal assays were performed by atomic absorption spectroscopy using an Instrumentation Laboratory AA/AE Spectrophotometer 551. The copper assays in preliminary experiments and those studies leading to the development of the high-salts medium, however, were done by measuring the absorbance of the diethyldithiocarbamate (DEDTC) complex at 433 nm (Hulanicki, 1967). A sample (0.02 to 0.2 mL) containing 0.01 to 0.1 µmol of metal, 5 mL of a freshly-prepared 0.1% aqueous solution of sodium DEDTC, and 5 mL of ethyl acetate was mixed thoroughly in a test tube and allowed to separate. The absorbance of the ethyl acetate layer containing standard or unknown metal concentrations was measured against a blank obtained by contacting ethyl acetate with the appropriately diluted DEDTC solution. One-centimeter quartz cells were used in a Coleman Hitachi 124 double-beam spectrophotometer. Beer's law was obeyed up to an absorbance of 0.7 with a molar extinction coefficient of 1.3×10^4 mol/L/cm. Metal-free as well as biomass-free controls were routinely examined, and they matched the blank and standard metal solutions respectively. Analysis of a variety of other metals using a similar system was investigated, and used for analyses reported in Table 3.3. The wavelengths and molar extinction coefficients for these assays are listed in

Table 2.3. All analyses were done in duplicate or triplicate.

Metal uptake by the biosorbent was determined as the difference between the initial and final concentrations of metal in solution. In solutions of final concentration greater than 3 mM the solids' metal content was also determined directly after boiling in concentrated nitric acid. Both the specific uptake (micromoles of metal bound per gram of dried biosorbent) and the biosorptive yield (specific uptake x biomass concentration in grams per litre of medium) were calculated. Metal uptake results were fitted to an equilibrium model assuming one or two types of sites using a BASIC computer program (Appendix 2).

2.3 Sulphite Oxidation Measurement of Oxygen Transfer

Oxygen transfer was measured at 25°C in a Versatec fermentor (Pegasus Industrial Specialities, Agincourt, Ont., Canada) with a 2-L jar, and a Vibra-Mixer with a standard 6.5-cm diameter mixing plate positioned at the top of the hemi-spherical bottom section of the fermentor. Two liters of reaction mixture containing 100 mL of a 0.2 M borax-cobalt buffer-catalyst solution (19.07 g $Na_2B_4O_7.10H_2O$ plus ~0.5 g $CoCl_2.6H_2O$ were diluted to 1 L, stirred at 25°C overnight and filtered) and 100 mL of a 0.2 M sodium sulphite solution (25.2 g Na_2SO_3/L) were prepared, and an additional 25 mL of water was added to compensate for the volume removed for the zero-time sample. The stirring and aeration conditions were set, and 5 min was allowed for the gas and solution to equilibrate (reach pseudo-steady-state). A bulb and 25-mL pipette which had been flushed with nitrogen was then used to remove the zero-time sample from ~10 cm below the surface. Three 5-mL samples were then mixed with 5 mL of a 0.02 M iodine-

iodide solution (2.5 g I_2 plus 12 g KI were dissolved in 1 L of water and filtered through sintered glass), and then titrated with a 0.005 M sodium thiosulphate solution (2.5 g $Na_2S_2O_3.5H_2O$ plus 0.2 g Na_2CO_3 dissolved in 2 L of boiled water) to a starch indicator end-point. The thiosulphate solution was standardized using potassium iodate. After 15-20 minutes a second 25-mL sample was removed from the fermentor and treated in the same manner. The rate of sulphite oxidation was determined as the difference between the initial and final concentrations divided by the time of reaction, and was twice the rate of oxygen consumption. This system resulted in a zero-order reaction during oxidation of more than 75% of the sulphite.

2.4 Chemical Analysis of Biosorbents and Extract Samples

Biosorbent samples were hydrolysed (0.02 g / 8.5 mL) in sealed tubes at 100°C under two sets of conditions determined by preliminary experiments to be optimum for various analyses (Appendix 1). Mild hydrolysis in 1.2 M HCl for 2 h preceded analysis of neutral and acidic sugars, while harsh hydrolysis in 6 M HCl for 10 h was necessary before analyses of protein and glucosamine were carried out. After hydrolysis the samples were dried *in vacuo* and then redissolved in water, the solids were removed by centrifugation, and the supernatants were diluted to 20 mL. Extract samples containing HNO_2 were purged with air for 5 h at room temperature before assaying, but this treatment was not sufficient to avoid interference during sugar assays which were very sensitive to nitrous acid. All colorimetric measurements were carried out in duplicate using 1-cm round test-tubes in a Beckman single-beam spectrophotometer.

2.4.1 Phosphate

Phosphate was measured directly in both mild and harsh hydrolysate samples. Extract samples were digested by wetting with 0.2 mL of 3:2 (vol/vol) concentrated H_2SO_4 and 70% $HClO_4$, and heating over electrical elements for 30 min. The syrup was then diluted 100 times with water and heated for 10 min at 100°C. The method of King (1932) was modified for smaller volumes and constant sample size. A 5-mL sample (<200 µM P) was mixed with 0.2 mL of 70% perchloric acid, 0.2 mL of 5% ammonium molybdate and 0.1 mL of 1-amino-2-napthol-4-sulphonic acid solution (Fisher Scientific, dry mixture containing sodium bisulphite and sodium sulphite, prepared as directed). The colour was allowed to develop for 15 minutes at room temperature and the absorbance was read at 660 nm. No interferences were encountered during this assay.

2.4.2 Neutral sugars

Neutral sugars were measured directly in both the mild hydrolysate and in the extract samples using anthrone (Maddy, 1976) with glucose as a standard. The reagent was prepared by dissolving 0.5 g of anthrone and 10 g of thiourea in a cooled sulphuric acid solution (concentrated sulphuric acid added to 280 mL of water to make up 1 L). It was stored at 5°C and used within 2 weeks. A 1-mL sample containing 20–200 µg sugar (glucose equivalent) was mixed with 5 mL of the reagent, heated for 15 minutes at 100°C, and cooled. The absorbance was measured at 620 nm and was linear with concentration over the range. Analyses of the nitrous acid extract samples were discounted because interference resulted in high readings.

2.4.3 Glucuronic acid

Glucuronic acid levels were determined in the mild hydrolysate and extract samples using carbazole (Bitter and Muir, 1961) with glucuronolactone as a standard. A 1-mL sample containing less than the equivalent of 40 µg of glucuronolactone was added carefully with constant mixing to 5 mL of a cold solution of 0.05 M sodium tetraborate in concentrated sulphuric acid, heated for 10 minutes at 100°C, and cooled. Carbazole was recrystallized from ethanol, and a 0.25% solution in ethanol was prepared as needed. A 0.2-mL portion was mixed with the sample, heated at 100°C for 15 minutes, cooled, and the absorbance was measured at 530 nm. The blanks were less than 0.025 absorbance, as recommended. More than 0.4 M chloride may suppress the colour, and nitrate may enhance the colour in this assay. Thus analysis of the 3 M LiCl extract samples was not carried out.

2.4.4 Protein

Protein was measured in the harsh hydrolysate and extract samples using the Folin phenol reagent (Lowry et al., 1951; Schluf, 1981) using bovine serum albumin as a standard. Stock solutions of (A) 2% (wt/vol) $CuSO_4$; (B) 4% (wt/vol) sodium potassium tartrate; and (C) 3% (wt/vol) sodium carbonate in 0.1 M NaOH (prepared in boiled water) were used to prepare freshly a solution containing 2 mL each of (A) and (B) and 98 mL of (C). A 5-mL portion of this was mixed with a 0.5-mL sample containing between 10 µg and 100 µg of protein. After 10 minutes it was mixed vigorously with 0.5 mL of phenol reagent (Folin-Ciocalteau diluted 1:1 with water) and after an additional 30 minutes the absorbance was measured at 700 nm.

Ninhydrin was also used to estimate quantities of glucosamine plus protein (Rosen, 1957). A 1-mL sample containing 0.02 μmol to 0.4 μmol amine groups was mixed with 0.5 mL of sodium cyanide in acetate buffer (20 mL of 0.01 M NaCN was diluted to 1 L with buffer prepared by diluting 360 g of sodium acetate trihydrate and 67 mL of glacial acetic acid to 1 L) and 0.5 mL of 3% ninhydrin in 2-methoxy ethanol, and heated at 100°C for 15 minutes. Immediately 5 mL of 1:1 isopropanol/water was added and the sample was mixed vigorously. After cooling to room temperature the absorbance was read at 570 nm.

No interference with either of the protein assays was evident.

2.4.5 Glucosamine

Glucosamine and HNO_2-reaction products were measured by high performance liquid chromatography (Beckman 332 gradient liquid chromatograph with a refractive index detector and a 22 cm Brownlee polypore CA column, 10 μm carrier bead size, at 80°C, eluted at 0.3 mL per minute with degassed water). Protein plus glucosamine was also estimated using ninhydrin (Section 2.4.4).

2.5 Alkali Titrations of Biosorbent Samples

Samples of the old HSM biosorbents (propagated for 45 h in the high salts medium) were finely ground in a ceramic ball mill and 0.1-g samples in 10 mL water were titrated slowly under nitrogen with 0.01 M NaOH using a Radiometer Titrigraph type SBR 2c and a Titrator type TTT 1b (Radiometer, Copenhagen, Denmark). The sodium hydroxide solution was prepared in boiled water from a 50% syrup, and standardized using oxalic acid and disodium ethylenediaminetetraacetic acid solutions. The equipment was adjusted so that the biomass was titrated in 6 to 8 hours.

3. RESULTS

3.1 Background Experiments to Establish Experimental Methods

The first objective of this work was to establish methods to propagate repeatably the biosorbents which were to be the subject of study. These methods had to be such that growth conditions were as carefully defined as possible and were amenable to controlled variation. Two aspects of this were the handling of the organisms and the preparation of a totally defined synthetic growth medium. Preparation and refinement of the growth medium is discussed in Sections 3.2 and 3.3.

3.1.1 Inoculum preparation

A spore inoculum was used in the majority of experiments because it was easily quantified using a counting chamber, and the growth pattern following inoculation with spores was repeatable as long as the multiple-transfer protocol described in Section 2.1.1 was followed and the inoculum level was constant. The latter was accomplished by checking spore concentrations before each inoculation. The importance of the series of transfers on solid media which preceded growth in any particular medium in suspension culture was most obvious when working with R. japonicus. This mould grew well and produced a thick

vegetative "turf" on both organic (neopeptone) and synthetic media, but produced very few sporangia on the organic medium. Five transfers were necessary before a consistent morphology was observed (ie, one that was the same on subsequent transfers) when changing either from the organic to the synthetic medium or vice versa. Some of the figures and tables presented in following sections were not obtained from a single experiment, but reproducibility of growth was such that results were compiled from independent experiments which were repeated with different overlapping sampling schedules.

In the course of experiments to devise preliminary defined media, R. javanicus, R. arrhizus, R. delemar var. multiplicisporus, R. oligosporus and R. oryzea all grew and sporulated well on solid media and their spores were readily recovered in water, which facilitated preparation of inocula. Of these, R. javanicus, R. arrhizus and R. oryzea exhibited good growth in suspension in shake flasks; they grew rapidly and formed a fine dispersion. Pellets were considered undesirable in this work because conditions in the interior would be unknown and the biomass could not be considered uniform. Most studies were carried out with R. javanicus, although trends were checked with other species (Section 3.3.1).

In general a spore inoculum undergoes a longer lag phase than a vegetative inoculum, but such an inoculum was not used in most of this work. The exception was the toxicity studies (Section 3.2.4.2) which were performed using a very young, disperse inoculum to mimic conditions of previous experiments. However, a mycelial inoculum is difficult to dispense repeatably

(particularily when working with an aseptate mould such as Rhizopus which is not amenable to partial homogenization before dispensing), and is not as easy to quantify rapidly as are spore and yeast inocula. Rapid assessment of the inoculum is necessary if its level is to be kept constant in a number of different experiments, and spore and yeast inocula may be counted.

Some species of Mucor were reported to exhibit yeast-mycelial dimorphism under a variety of conditions (Rogers et al., 1974), so the possibility of propagating a yeast-like vegetative inoculum of R. javanicus was investigated using 50 mL of synthetic medium A in 150 mL sealed Wheaton vials. The composition was adjusted to include up to 130 g glucose, 20 g ethanol or 100 g calcium carbonate per litre of medium. The calcium carbonate did not dissolve completely, but resulted in increased pressure and CO_2 levels in the gas space as acid was produced. The vials were sealed when hot, cooled and inoculated with 10^7 spores. The oxygen level was adjusted in one of three ways; the medium and gas space was either left undisturbed, was initially flushed with nitrogen, or was vented daily to release excess gas before 1 mL of sterile air was added. No growth was observed in the flasks which had been flushed with nitrogen, and no evidence of yeast-like growth was observed under any of the other conditions, even when growth was severely limited by the O_2 supply. In the bottles to which ethanol had been added small strong pellets formed, although growth was slower than in the other cultures under the same conditions of aeration. Since disperse growth was desired, this observation was not pursued further.

Table 3.1

Effect of Acid Treatment of R. javanicus on Subsequent Cu^{2+} Uptake and Biomass Viability

Biomass treatment before water-washing	Cu^{2+} Concentration Initial (mM)	Final (mM)	Uptake (μmol/g)	Final Solution pH
A Acid wash (pH 1)*	2.0	1.16	210	3.1
B Acid wash (pH 2)*	2.0	1.05	230	3.5
C Water wash	2.0	1.10	220	3.6
A	1.0	0.36	160	3.2
B	1.0	0.38	160	3.8
C	1.0	0.37	160	3.8
A	0.2	0.11	68	3.4
B	0.2	- growth of mould -		
C	0.2	- growth of mould -		

* pH 1 corresponds to 0.1 M HCl; pH 2 corresponds to 0.01 M HCl

Biomass was from fermentation #2, Table 3.7

3.1.2 Biosorbent preparation

It was necessary to treat the biomass before drying, to prepare biosorbents for subsequent analysis of metal uptake. The biosorbents (cell walls) had to be "clean" so they would not release soluble compounds which might affect the metal uptake determination. Possible components include residual cytoplasm which could complex the metal in solution. As well, metals sequestered from the growth medium could result in reduced uptake of the ion of interest or cause release of a large number of unknown metals into the contacting solution, which might interfere with analysis. For these reasons it was decided that the biomass should be filtered and water-washed to remove the majority of the nutrient broth, and then homogenized in water in a Waring blender, filtered, resuspended in the blender in acid, filtered, and then repeatedly resuspended in water and filtered until a clear, colourless, neutral filtrate was obtained. This usually involved about ten water-washes. Preliminary water-washing and homogenization resulted in a large proportion of hyphal ghosts, leaving only some tips intact where they were separated from the larger mass by septa. Subsequent washing with hydrochloric acid, resuspension and water-washing resulted in elimination of intact hyphal segments. In an investigation into the effects of acid strength on subsequent copper uptake (Table 3.1), the pH of the acid wash did not greatly affect the uptake at final copper concentrations of 0.4 mM and 1.1 mM, although the final pH of the copper solution was lower when the copper uptake was higher (increased exchange with hydronium ions occurred; Treen-Sears et al., 1984). However, an acid concentration of at least 0.1 M was

needed to kill the mould (Table 3.1). The acid caused a marked softening of the biomass structure (the filter-cake was much more compact and offered a greater resistance to flow) and made the intact segments susceptible to homogenization.

3.1.3 Metal uptake determination

Small pieces of unground biosorbent (less than 1 mm smallest dimension) were used in the metal uptake assays. This facilitated the separation of the solids and the liquids (it obviated the need to filter the liquid), and was more realistic than using finely ground biomass in that any commercial biosorbent would probably be pelletized. In preliminary experiments copper uptake by two types of biosorbents was found to be rapid initially as uptake equivalent to 70% of the final value occurred within the first half hour (Figure 3.1). Equilibrium was reached within 10 hours or less, so the standard contact time of 15 hours which was adopted for routine assays was ample.

Initially the copper solutions were of neutral pH, but the pH dropped to 3-3.5 as metal ions exchanged with hydrogen ions on the acid-washed biosorbent (Treen-Sears et al., 1984). The final pH of the contacting solutions was routinely measured, although it was not adjusted, nor was it controlled with buffers. Some samples left for more than 24 hours were found under microscopic examination to be extensively contaminated with bacteria, particularily at lower metal concentrations. This was accompanied by elevated pH levels and/or cloudiness of the solution, as well as anomalously high values of metal uptake. All samples were therefore checked microscopically for evidence of microbiological activity and the final solution pH was

Figure 3.1: Approach to Equilibrium During Metal Uptake Assay

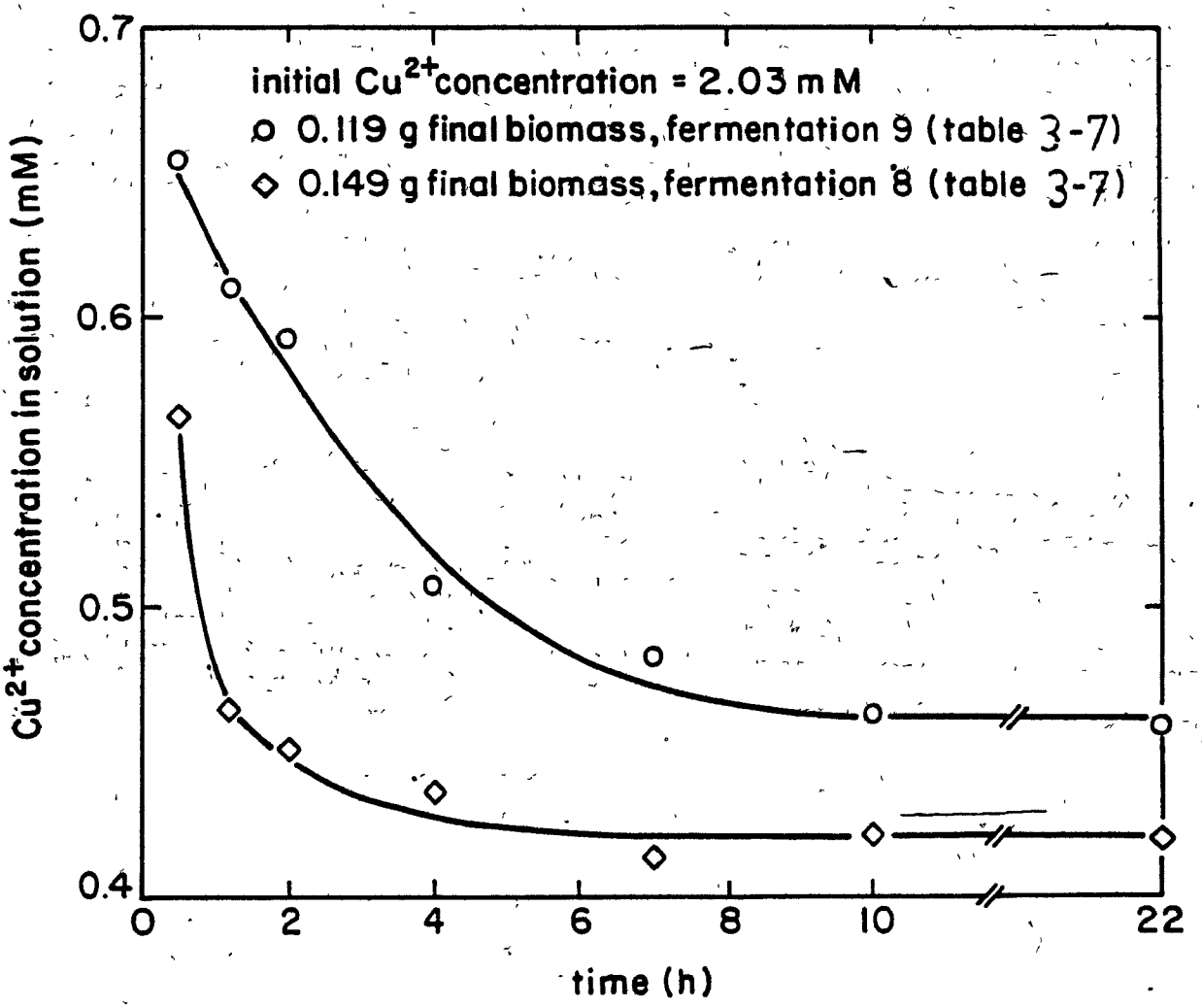


Table 3.2

Effect of Biosorbent "Concentration" on

Cu^{2+} Uptake by *R. javanicus*

Biosorbent Mass (g)	Cu^{2+} Concentration Initial (mM)	Final (mM)	Cu^{2+} Uptake (µmol/g)
0.0999	1.99	1.55	110
0.1006	1.99	1.55	109
0.2050	3.19	2.26	113
0.2033	3.19	2.33	106
0.0519	1.40	1.16	112
0.0489	1.40	1.19	103

The biosorbent was prepared from stationary-phase biomass propagated without pH control in medium A in Versatec fermentors

measured. No bacterial contamination was detected with the 15 hour contact time routinely used.

The ratio of biomass weight to solution volume in the metal uptake determinations was found not to affect the saturation equilibrium metal uptake value (Table 3.2). This indicates that the methods used to clean the biomass were adequate because release of interfering substances from different quantities of biosorbent in the same volume of solution would otherwise have caused a trend of decreasing uptake with increasing mass of biosorbent in suspension. Analysis of the limited data in Table 3.2 reveals that the standard deviation of the entire metal uptake determination under this variety of conditions was 3%. Sources of error occurred in every step of the assay, including the metal concentration determinations, weighing of the biomass and measurement of the liquid volume. In most cases the metal uptake was determined as the difference between the initial and final metal concentrations (although, above ~2 mM final metal concentration this was checked by analysis of the bound metal after hydrolysis of the biomass) so the error in the determination depended upon both the accuracy of the individual metal concentration determinations and the relative magnitudes of the initial and final concentrations. For a single determination the diethyldithiocarbamate assay was accurate to within 3% and the atomic absorption method was accurate to within 4% at the lowest concentrations and 1% at higher concentrations. This was improved to 0.5 - 2% by using the average of from two to five measurements. The biosorbent weight was determined to within +/- 0.0003 g, so at lower concentrations in suspension the error

was proportionately greater, approaching 1% with 0.05 g biosorbent in suspension. The metal solution was dispensed with automatic pipettes which were accurate to within 0.2%, but repeated dispensing (up to 5 times) meant that the error was in fact 1% (the precision was much better than the accuracy). The same errors must be applied to the dilution of the metal solutions prior to analysis. Combining these errors, the metal uptake determination was accurate to within 3%. Additional sources of error were imperfect preparation of the biosorbents, leading to interferences in the metal determinations, as well as many other indeterminate factors which are common in wet chemical analyses.

Estimation of the metal sequestered on the biomass by analysis of the hydrolysate was subject to errors which were less readily quantified because the rinsing of the biomass was a poorly controlled procedure. Removal of the interstitial metal-containing solution without desorbing anything from the biomass was clearly impossible, and the best that could be hoped for was a compromise. These determinations agreed within 10% with the measurements by difference at metal concentrations of 1-2 mM, so they were accepted.

Table 3.3

Uptake of a Variety of Metals by

R. javanicus and *R. arrhizus* Biosorbents

		*R. javanicus**		*R. arrhizus***
Ion	pH	Final Metal Concentration (mM)	Uptake (μmol/g)	Saturation Uptake (μmol/g)
Co^{2+}	3.3	0.38	320	-
Cu^{2+}	3.1	0.80	300	250
Ni^{2+}	3.4	0.42	290	-
Zn^{2+}	3.4	0.42	280	300
Cd^{2+}	3.3	0.68	160	270
Ag^{+}	3.2	0.77	140	500
Pb^{2+}	2.9	0.42	320	500

* *R. javanicus* was propagated in medium A in shake flasks (50 mL/250-mL flask)

** *R. arrhizus* uptake was measured at pH = 3.5 - 4.0 (Tobin *et al.*, 1984)

Sequestering of a variety of metals of possible commercial interest, or of interest because of their complex chemistry, was examined. Uptake measurements for R. javanicus biosorbent grown in medium A in shake-flasks are compared with saturation values for R. arrhizus samples (Tobin et al., 1984) in Table 3.3. Comparison is not straightforward because no strength of binding or other mathematical model parameter was reported for the R. arrhizus biosorbent, so uptake at comparable solution concentrations could not be calculated. However, assuming that a large fraction of the maximum possible uptake occurred with the R. javanicus sample, the results were similar for copper and zinc, while the cadmium, lead and silver uptake values were much higher for the R. arrhizus biosorbent. The low value of silver uptake for the R. javanicus biosorbent indicates that very little of the chloride introduced during the acid-washing remained because any residue would contribute to the immobilization of the silver in the form of extremely insoluble silver chloride.

Copper was chosen for subsequent experiments to examine the effects of growth conditions on biosorbent production and quality because it has a simple solution chemistry at the pH of the biosorption assays, and it was taken up to a similar extent by both species. These facts suggest that the copper was taken up by a common process which was directly attributable to the biosorbent composition rather than to other factors (ie. ion exchange rather than collection of colloidal substances or adsorption was occurring). As well, the diethyldithiocarbamate assay of copper was less susceptible to interference from other metals (Table 2.3).

3.2 Effects of Medium Composition and Growth Conditions on Biosorbent Production

Once reliable experimental methods were established, the effects of various medium components and growth conditions on the quantity of biosorbent produced were investigated. Generally, the term "biosorbent" refers to the material used for metal uptake, while "biomass" refers to the living organism in suspension culture. However, to avoid confusion with the conditions used in metal uptake experiments and to emphasize that reference is being made to growth experiments, "biomass concentration" refers to the biosorbent recovered from the biomass in a unit volume of growth medium. The biosorbent was highly refined and consisted chiefly of cell wall material, so the values determined in this work represent only a small fraction of the quantity which one would find using a conventional dry weight assay of whole cells (Section 3.4.1). Thus it must be emphasized that the figures given for biomass concentrations in cultures cannot be compared directly with literature values.

Biomass production was not in itself the aim of this work; clearly production of smaller quantities of a very effective metal sequestering agent is preferable to propagation of larger amounts of material which does not possess the desired metal uptake characteristics. The quantity "biosorptive yield", which is the product of the biomass concentration and the metal uptake capacity at a given metal concentration in solution was used to assess biosorbent production. Relatively high solution metal concentrations were used so the biosorbent would be close to

saturation and this would be more a measure of binding capacity than strength of binding. However, it has subsequently been found that the measurement of low values of biosorptive yield at concentrations in the neighbourhood of 2 mM may signify low binding strength. For comparison of biosorbent samples metal uptake was modelled as an equilibrium between the solution and sites of complexation within the solid (Treen-Sears et al., 1984). Unless uptake is measured over several orders of magnitude, calculations assuming only one type of site are possible. Thus the number of sites (W, μmol/g) and the apparent strength of binding (k, mM^{-1}) are frequently quoted, although it is recognized that in such a complex material they represent an average value and do not necessarily correspond to a single type of site.

The first media on which the Rhizopus species were grown (media A, A2 and B; Table 2.2) were based on a variety of less well defined media and information regarding the mineral requirements of fungi (Bowen, 1979). This Section deals with how to propagate effective biosorbent in concentrated suspension. Preliminary investigations of the effects of medium composition and growth conditions on biosorbent production pointed out that very little biomass was being produced, but investigations of the role of the cation content of the medium led to the development of a high-salts medium for copious production of R. javanicus biosorbent. Studies of the effect of cations in the growth medium on biosorbent quality are related in Section 3.3.

3.2.1 Effects of "macro" medium components and pH on biosorbent production

The components in the growth medium present in the highest concentrations were the first to be examined.

The effect of the carbon source on the production of Rhizopus biosorbents was not investigated; the only studies which were carried out involving carbon in the medium were related to the possibility of growth of a yeast-like inoculum (Section 3.1.1). It was found that glucose levels up to 130 g/L did not inhibit growth, and that when ethanol (20 g/L) was added to the medium small (1-2 mm), strong pellets were produced. Rhizopus species are known to grow vigorously on a large number of soluble and insoluble substrates (Inui et al. 1965), so the question of the carbon source should be approached with economics in mind, and in the absence of direction on this issue, investigations would have been intractable and of dubious practical use. Glucose was readily assimilated by all of the species studied so it was used in these investigations.

All cultures studied were typical of the genus in that they did not grow with nitrate as the sole nitrogen source (Inui et al., 1965). This was confirmed in the course of early attempts to devise a synthetic growth medium. All species grew well on ammonium salts or urea, however, and the latter was chosen for subsequent work to avoid accumulation of acid as ammonia was removed from solution.

Table 3.4

Effect of $MgSO_4$ Levels on Biosorbent Production in Medium A

Growth Medium		Biosorption		
$MgSO_4$ Concentration (g/L)	Biomass Concentration (g/L)	Final Cu^{2+} Concentration (mM)	Cu^{2+} Uptake (μmol/g)	Biosorptive Yield (μmol/L)
0.25	2.2	2.31	170	370
0.5	2.3	2.41	150	350
0.75	2.0	2.7	160	320
1.0	1.7	2.4	200	340

Biomass was propagated for 56 h in 50 mL of medium in Erlenmeyer flasks. Final pH in the growth medium = 2.

Table 3.5

Effects of Phosphate Buffering on Biosorbent Production in Medium A

Growth Conditions			Biosorption		
K_2HPO_4/PO_4* (mol/mol)	Initial Medium pH	Biomass Concentration (g/L)	Final Cu^{2+} Concentration (mM)	Cu^{2+} Uptake (μmol/g)	Biosorptive Yield (μmol/L)
0.0	4	2.3	2.4	140	320
0.2	4.5	2.2	2.1	160	360
0.8	6	1.8	2.3	230	410
1.0	6	1.7	2.3	280	480

* balance KH_2PO_4; levels equivalent to 0.5 g/L KH_2PO_4

Biomass was propagated for 68 h in 50 mL medium in Erlenmeyer flasks. Harvesting occurred at least 12 h after growth medium reached pH 2.

The effects of mineral concentrations in the growth medium on biosorbent production were studied in a preliminary manner, and then returned to later in the development of the research.

The effects of magnesium sulphate levels were studied in variations of medium A, but little difference was observed in biosorbent production in shake-flasks in which the media $MgSO_4$ concentrations were increased from 0.25 g/L to 1 g/L (Table 3.4).

Calcium is reported not to be essential to fungi (Bowen, 1979), and in some early experiments Rhizopus species were successfully grown on media containing no added calcium. However, biomass production was improved 25% in the presence of calcium chloride (1 mg/L) in medium A so it was thereafter included. Growth was not improved upon addition of boron or vanadium, which are also reported not to be essential (ibid), so they were not included in subsequent medium formulations.

When K_2HPO_4 was the only source of phosphate in medium A, both biomass production and Cu^{2+} uptake improved as the salt concentration was increased from 0.5 g/L to 2 g/L (Figure 3.2). The effect of buffering by the phosphate was examined by varying the ratio of the mono- to di-hydrogen salt, keeping the phosphate level constant (Table 3.5). This resulted in different initial pH values. The maximum biomass production occurred in the medium with the lowest initial pH (4). However, the optimum biosorbent production occurred in the medium with the highest proportion of the dipotassium salt, which offerred the maximum buffering capacity as the mould produced acid. Thus the biosorbent production was related to the medium pH and buffering capacity rather than to the phosphate level.

Figure 3.2: Effect of K_2HPO_4 Concentration on Biomass Production and Cu^{2+} Uptake

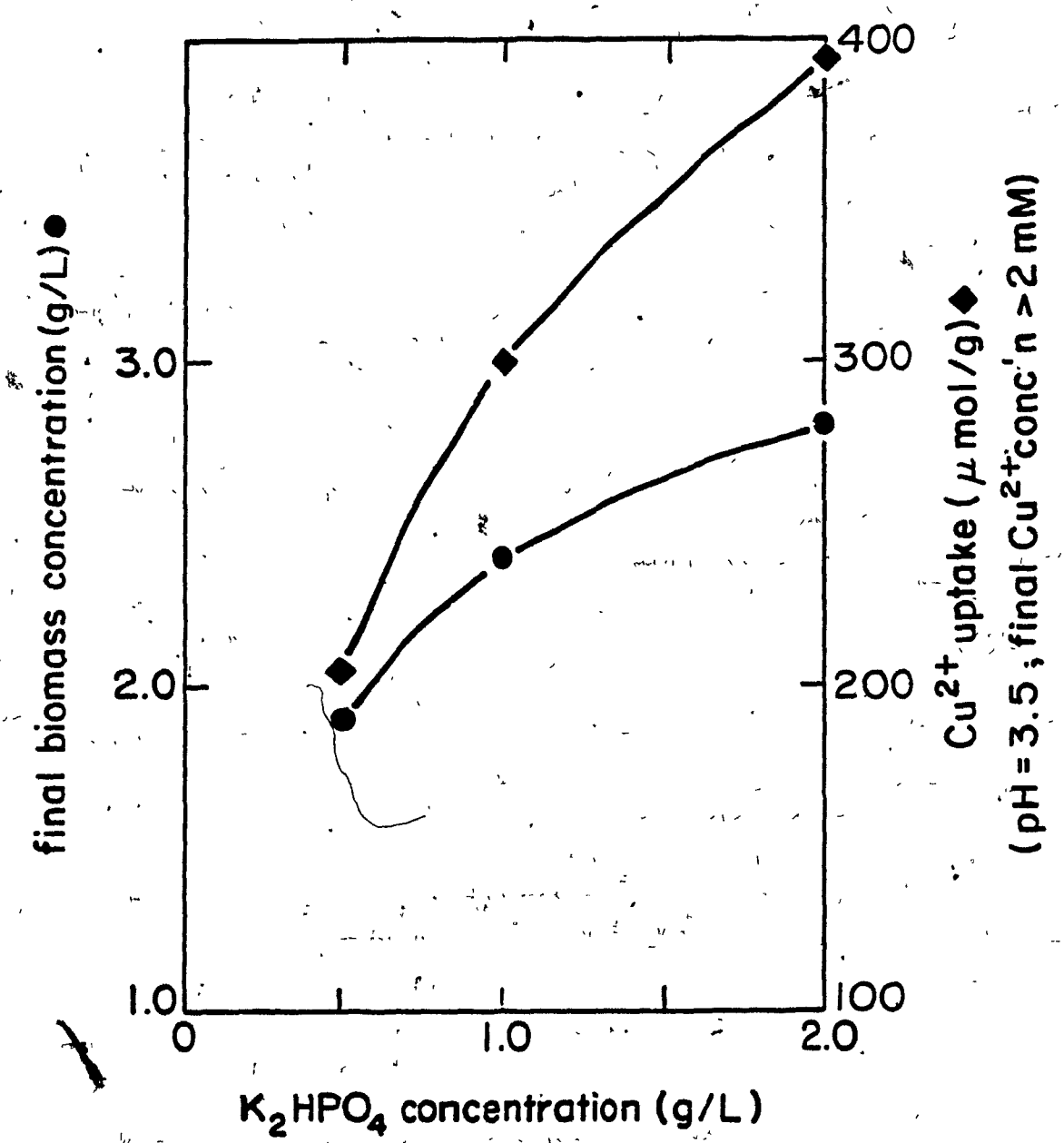


Table 3.6

Variation of Copper Uptake and Biosorptive Yield with Time of Propagation of *R. javanicus* in Medium A2 in Shake Flasks

Growth Conditions			Biosorption		
Time (h)	Biomass Concentration (g/L)	Medium pH	Final Cu^{2+} Concentration (mM)	Cu^{2+} Uptake (µmol/g)	Biosorptive Yield (µmol/L)
15	0.048	4.3	3.4	900	43
			1.5	450	22
16	0.097	4.3	1.4	340	33
19	0.28	4.3	1.4	145	41
20	0.17	4.3	3.4	410	70
			1.7	360	58
22	0.36	3.8	1.3	160	58
24	0.34	3.6	3.5	290	98
			1.6	220	75
26	0.48	3.2	1.3	180	86
27	0.49	3.5	3.6	210	100
			1.7	170	83
28	0.68	2.9	1.2	160	110

Biomass was propagated in 100 mL of medium in Erlenmeyer flasks.

These results are from two experiments; results for 15, 20, 24, and 27 h were from an experiment with an inoculum of 1.2×10^8 spores/L and the rest were from one with 1.4×10^8 spores/L.

The dynamics of growth and biosorbent production by R. javanicus were studied in a preliminary manner in medium A2 (similar to medium A with high levels of the trace metals but lower levels of $MgSO_4$, KH_2PO_4 and urea; Table 2.1) in shake flasks (Table 3.6). These results are the worst example of reproducibility. This most likely occurred because of the formation of fairly large pellets (several millimeters in diameter) with the rather low inoculum levels. The copper uptake of the resultant biosorbent samples at a concentration of 3.4 mM dropped from 900 µmol/g very early in the fermentation to as little as 200 µmol/g, and the biosorptive yield stabilized at ~100 µmol/L as growth slowed and the medium pH dropped. The biosorptive yield (at copper concentrations between 1.2 and 1.7 mM) increased slowly as the growth rate declined.

Growth of R. javanicus in a variety of media under several different growth conditions was followed during the course of nine growth experiments in Versatec fermentors (Table 3.7). In the following paragraphs, run numbers to which the observations refer are quoted in parentheses.

The time required to reach 0.1 g/L biomass concentration was related to both the lag during germination of the spore inoculum and the inoculum concentration. The lag was extended if the pH was 4 or 5.5-6 rather than the optimum pH 5 (runs 4, 6-8) or in the presence of 2% CO_2 in the gas stream (runs 8 and 9).

Table 3.7

Summary of *R. javanicus* Growth in "Versatec" Fermentors

Run #	Medium Composition	pH	Air Flowrate (L/min)	Inoculum (spores/L)	Time to 0.1 g/L Biomass (h)	Specific Growth Rate (h^{-1})	Time of Initial NaOH Demand (h)	Time of Growth Halt (h)	Final Biomass Conc'n (g/L)	Post-growth Consumption Glucose (mmol/L/h)	Post-growth Consumption NaOH (mmol/L/h)
1	A + 1.7 g/L Na_2SO_4[d]	5.5	1.0	$5x10^8$	-	0.05	42	45	1.1	0.92	0.37
2	"	4	1.0	"	-	0.05	44	47	0.82	0.59	0.16
3	A + 0.3 g/L NaCl[d]	5.5	0.4	(10^7)	29	0.08	30	45	0.46	0.4	0.18
4	B	5.4	4.0	$3x10^9$	24	0.1	29	35	0.41	-	-
5	A	5	"	"	16	0.08	-	37	0.85	-	-
6	B	4	0.5>4[a]	$5x10^7$	20	0.16[b]	24	25[b]	0.42	-	-
7	B	6	"	"	24	0.13[b]	35	36[b]	0.35	-	-
8	B	5	3.0	$4x10^8$	14	0.16	23	24	0.44	0.53	0.28
9	B	5	3.0[c]	"	24	0.13	32	36	0.38	1.0	0.77

a aeration increased after formation of spore-bodies
b growth rate varied as aeration changed
c 2% CO_2 added to air
d additional salts had little effect on growth and biosorbent production (Section 3.2.1)
- not determined

Propagation was carried out in 1.5 L of medium.

Specific growth rate was calculated as the slope of a semi-logarithmic plot of the growth curve; 5-8 biomass determinations were carried out in each case.

Once the spores germinated, hyphae grew rapidly, branched, and formed a cottony mycelium as the biomass increased exponentially. The specific growth rate did not always increase as the lag time decreased (runs 3-9), indicating that factors inhibiting germination did not necessarily influence growth and vice-versa. The initial pH of the medium affected the growth rate during exponential growth, with a maximum rate being observed at pH ~5 in both media (runs 1, 2, 5 in medium A; runs 4, 6-8 in medium B). A lower growth rate (0.08 h^{-1}) was observed in medium A than in the more dilute medium B (0.16 h^{-1}). In both media, the rates were about 25% lower at pH 4 and 6 than at pH 5.

At a very low biomass concentration (usually less than 1 g/L), growth stopped and acid production began. Thickened hyphae, septa and large globular spore bodies formed, and the culture broth exhibited an increased tendency to foam. When the pH was controlled, acid was produced and glucose was consumed at constant rates which were proportional to the biomass concentration, although acid equivalents were produced faster and more efficiently at higher pH (runs 1, 2) and at elevated levels of CO_2 (runs 8, 9). Under pH control the biomass concentration changed little during the 10 to 20 h after the commencement of acid production, whereas without pH control, the biomass increased slowly and some spore bodies germinated.

Table 3.8: Effect of Light on Growth and Subsequent Cu^{2+} Uptake by *R. javanicus*

Condition	Growth		Biosorption		
	Time (h)	Biomass Concentration (g/L)	Copper Concentration (mM)	Uptake (µmol/g)	Biosorptive Yield (µmol/L)
light	25	0.168	1.71	240	41
dark	25	0.133	1.74	280	38
light	27	0.241	1.47	370	90
dark	27	0.187	1.63	330	63
light	32	0.577	1.28	340	200
dark	32	0.369	1.42	390	140
dark	34	0.420	1.31	420	180

initial copper concentration = 1.98 mM

3.2.2 Effects of Light on Biosorbent Production

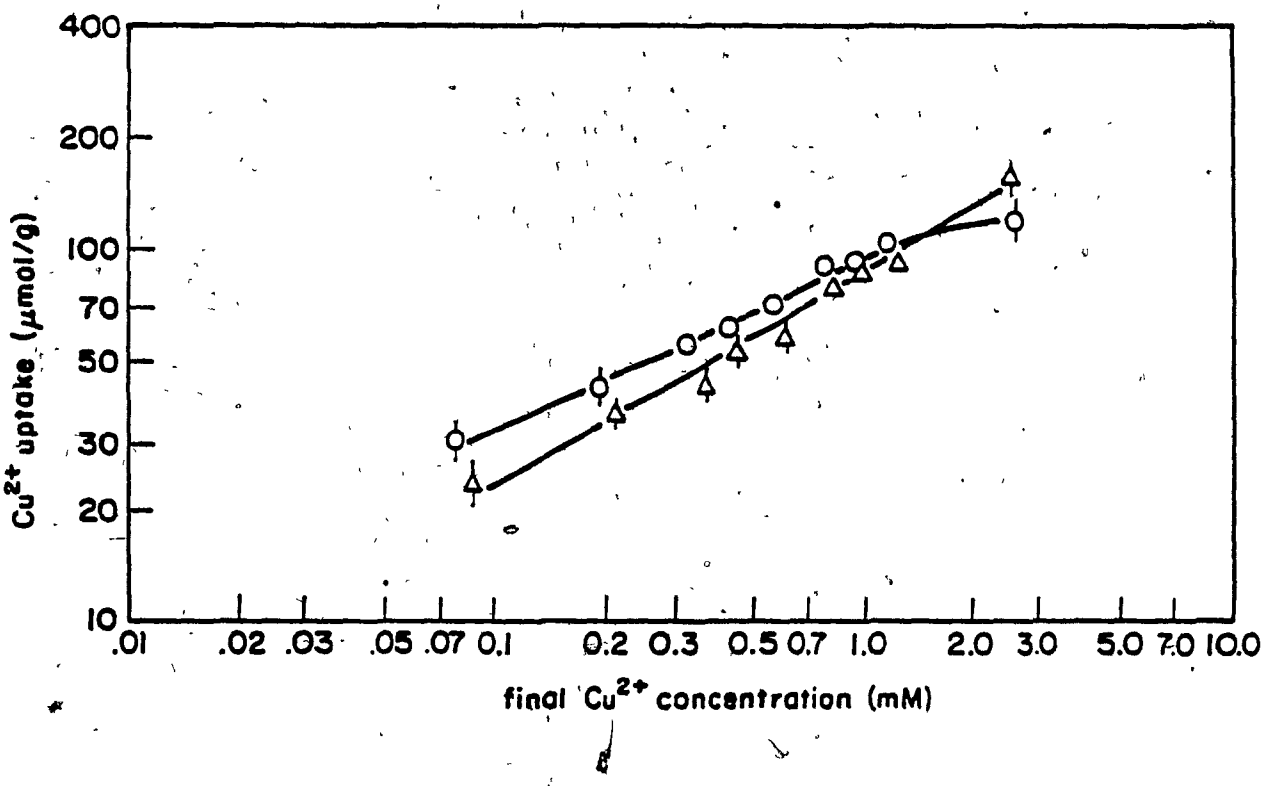
Light is known to stimulate growth of Phycomycetes (Bergman et al., 1969; Raudaskoski and Viitanen, 1982), and on solid media Rhizopus sporangiophores were found to be phototropic and light was necessary for differentiation leading to profuse sporulation. Most studies in fermentors were carried out under the normal low laboratory light but the effect of light on growth in suspension was investigated in two Versatec fermentors, one of which was wrapped in foil while the other was exposed to a bank of six fluorescent tubes.

In two replicate experiments, growth in medium B at pH 5 was more rapid in the lighted fermentor (specific growth rates were 0.22 h^{-1} and 0.23 h^{-1}) than in the wrapped fermentor (0.14 h^{-1} and 0.16 h^{-1}). The light did not affect the spore germination lag period, but did affect the copper uptake behaviour. Light caused an increase in the biosorptive yield at any particular time during the fermentation (Table 3.8), although the copper uptake was not constant; it went through a maximum which was observed earlier for the biosorbent from the illuminated fermentor. Illumination also affected the shapes of the Cu^{2+} uptake curves for the final biomass samples harvested after the commencement of acid production in such a way that the strength of copper binding was decreased while the number of uptake sites was increased (Figure 3.3). The standard growth conditions in the fermentors under low light levels led to a specific growth rate similar to that observed when the material was propagated in the dark (run 8, Table 3.7).

Figure 3.3: Copper Uptake by R. javanicus Biosorbents Propagated in the Dark and the Light

O dark -- harvested after 44 h of growth

Δ light -- harvested after 34 h of growth



3.2.3 Effects of aeration on biosorbent production

Oxygen supply to submerged cultures of aerophilic organisms is crucial to their successful and rapid growth, and in the Versatec fermentors it was found that the air flowrate affected both biomass and acid production; at low air flowrates (0.33 vol/vol/min) acid production began at lower biomass concentrations (runs 6, 7 Table 3.7). In fermentations with variable air flowrates the rate was increased to 1.3 vol/vol/min once alkali was demanded, which caused a rapid halt to acid production, germination of the spore bodies which had formed and recommencement of growth. This cycle was later repeated when the aeration rate was increased to 2.7 vol/vol/min. Unless stated otherwise this maximum air flowrate was used in all growth studies. There was no evidence of oxygen limitation under this condition.

Air dispersion, flow patterns and gas retention in the Versatec fermentors varied dramatically under different conditions of air flowrate and agitator power observed during the course of routine work), so oxygen transfer in the Vibra-Mixer system was examined. Oxygen transfer was studied using sulphite oxidation (Figure 3.4) using a reaction mixture with cobalt catalyst and borate buffer which exhibited a constant reaction rate at both low and high oxygen transfer rates over greater than 75% of the possible range of sulphite concentrations. Three important flow regimes were observed in the fermentor: at low power (<30 W) the air was poorly dispersed and oxygen transfer was slow because the gas-liquid interfacial area was small (the bottom surface of the gas trapped under the

mixing plate was relatively smooth and the bubble size was very large); at intermediate power (~ 70 W) the gas trapped under the plate was broken up but there was little pumping action so the bubbles went straight up and the gas retention in the bulk of the fluid was relatively low; at high power there was greater dispersion of the gas (the bubble size was smaller) and greater pumping of the fluid (the bulk was increasingly filled with bubbles and the gas retention was increased). At all power settings which resulted in dispersion of the gas the oxygen transfer rate increased with air flow rate until the agitator plate could not disperse the gas quickly enough and started to become "flooded". A slight drop in the oxygen transfer rate occurred at this point because of the increased bubble size, but the rate then continued to rise as the air flow rate was increased. At low power settings the flooding of the plate was more serious and was characterized by large bubble formation.

During growth of the mould the agitators were maintained on full power and only the air flowrate was varied. This strategy was adopted because hyphal suspensions require high shear rates to maintain gas dispersion (Brierely and Steel, 1959) and the Vibra-Mixer is an inherently low-shear mixing apparatus. At maximum power and air flowrates <4.5 L/min the bubbles in the aqueous sulphite reaction mixture were finely dispersed. More power would be required to disperse gas in a thick mycelial suspension, but little flooding of the agitator plate in the fermentors containing the growing mould occurred as there was no evidence of large bubble formation.

The effects of aeration and inoculum size on biosorbent production in Erlenmeyer flasks containing either 50 mL or 100 mL of medium B were also examined (Figure 3.5). The surface to volume ratios were approximately 0.7 and 0.3 cm^{-2} respectively. Oxygen limitation was suspected because the flasks with the higher medium volume smelled strongly of alcohol and esters (Jones and Greenfield, 1982). A spore concentration of 7×10^7 spores/L led to maximum production of pelletized biomass after 48 h in a broth of final pH~2. In flasks containing 100 mL of broth the mould initially grew more slowly than it did in flasks containing 50 mL, but the final biomass concentrations were higher. The pH remained constant for the first day then fell slowly in the flasks containing 50 mL and more rapidly in the flasks with 100 mL of medium. The biomass from the higher medium volume also exhibited higher copper uptake, so the biosorptive yield was higher in the flasks containing the larger volume of medium.

Figure 3.4: Sulphite Oxidation Measurement of Oxygen Transfer in the "Versatec" Fermentor

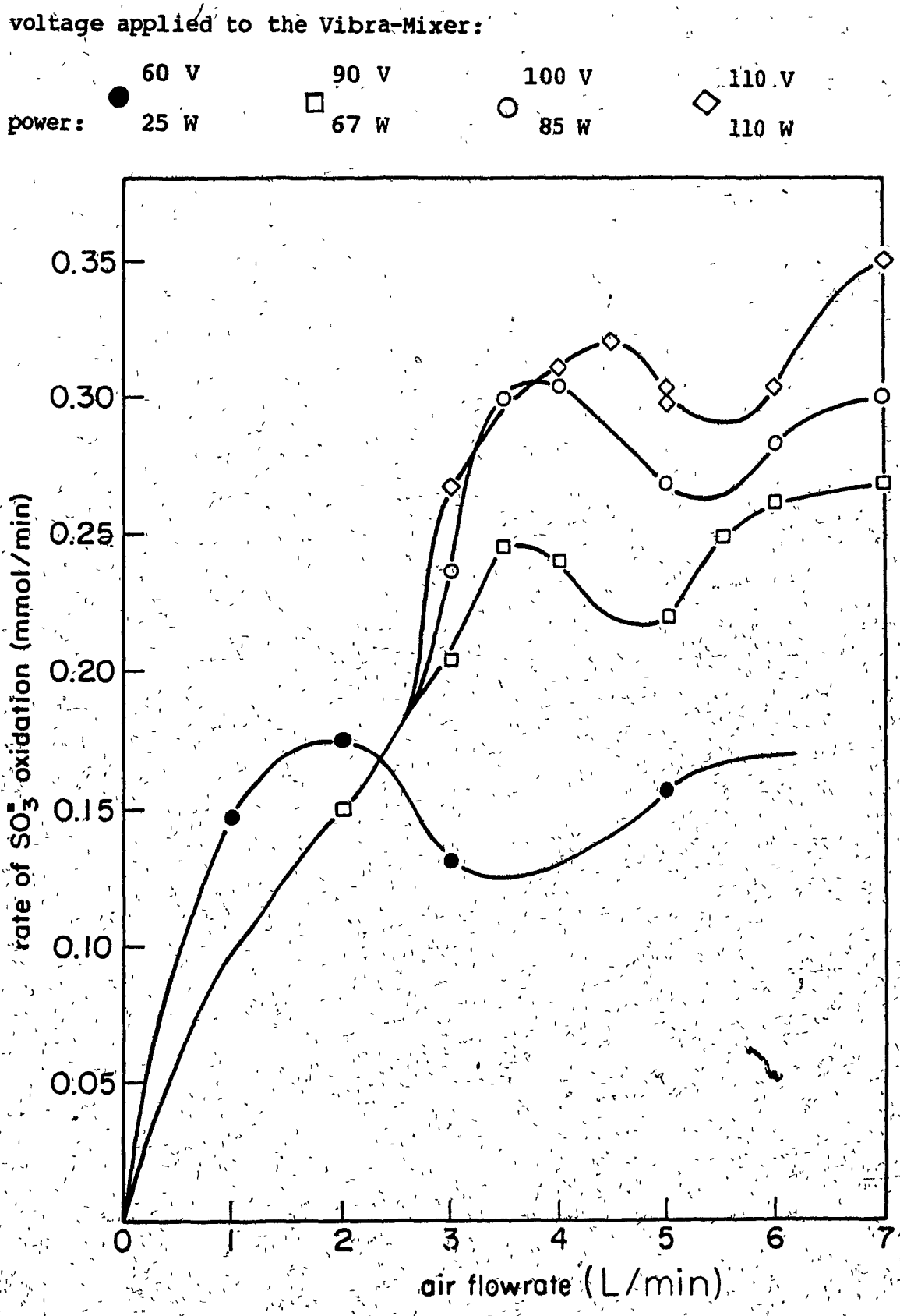


Figure 3.5: Effect of Inoculum Size and Aeration on Biosorbent Production in Shake Flasks

solid figures -- 50 mL of medium / 250-mL flask

open figures -- 100 mL of medium / 250-mL flask

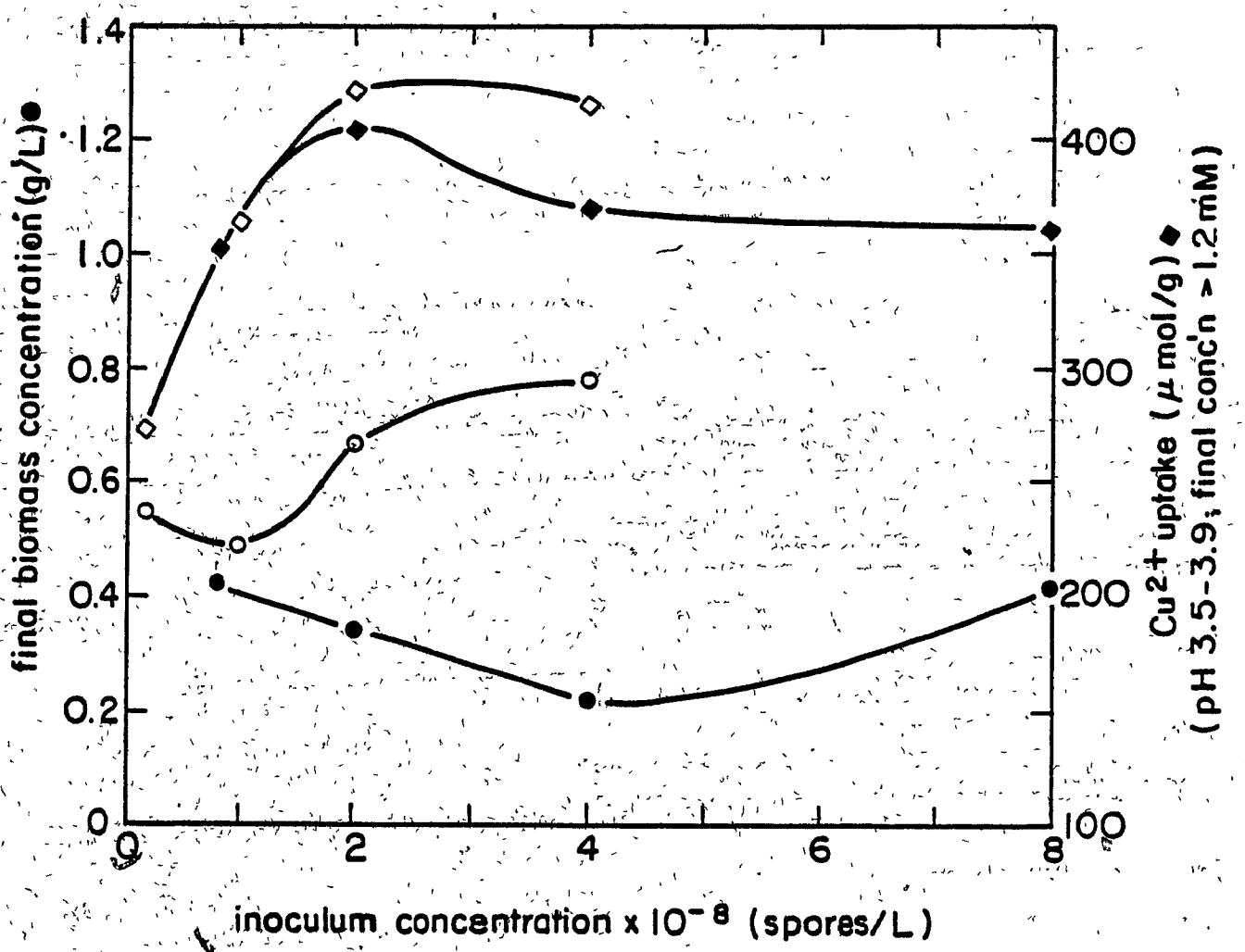


Table 3.9

Variation of Cu^{2+} Uptake and Final Biomass Concentration in Media A and B

Medium	Biomass Concentration (g/L)	Copper Uptake (µmol/g)	Biosorptive Yield (µmol/L)
A (pH 5)	0.82	270	220
A (pH 6)	1.1	210	230
B (pH 5)	0.35	220	77
B (pH 6)	0.42	180	76

Biomass was propagated in Versatec fermentors and harvested at the commencement of acid production

Copper uptake was determined at pH 3, with final copper concentrations of 1 mM for the biomass propagated at pH 5 and 1.2 mM for the biomass propagated at pH 6.

3.2.4 Development of the High-Salts Medium for Growth of *R. javanicus*

3.2.4.1 Effects of addition of a mixture of metals to the growing cultures

As discussed in Sections 3.2.1 and 3.2.2, growth in both media A and B was fairly rapid (specific growth rates up to 0.23 h^{-1} were observed), but biomass production was limited as growth stopped at low biomass concentrations (usually less than 1 g/L) and acid production commenced. Moreover, variation of the concentrations of individual macro-components of the medium or the aeration rate did not greatly affect either the final biomass concentration or the biosorptive yield; all changes were much less than an order of magnitude. When propagated in "Versatec" fermentors, final biomass concentrations and copper uptake values for the biosorbents varied inversely such that the biosorptive yield, at final copper concentrations of 1 to 1.2 mM, for a given medium remained constant both at pH 5, at which the most rapid growth was observed, and at pH 6 (Table 3.9). The biosorptive yield from medium A was approximately three times that from medium B and was proportional to the concentrations of iron, calcium, and trace metals in the media.

These results, in particular the observed strong dependence of the biosorptive yield on medium composition, indicated that essential trace minerals were being made unavailable for growth, possibly as they were being sequestered by the biomass. To test this hypothesis twice the normal amounts of minerals (Ca, Fe, Mn, Zn, Cr, Mo, Co, Ni, Cu) were initially added to medium B. The broth was further supplemented with the original quantities of these elements at different times during growth in 100 mL of

medium in shake flasks (Figure 3.6). When three additions of trace metals were made after spore germination but before commencement of acid production, growth continued without the formation of spore bodies, acid production was delayed, and the biosorptive yield was increased from 150 µmol/L to 250 µmol/L. This experiment was repeated in Versatec fermentors, with earlier and more frequent sampling (Figure 3.7). By gradually increasing the trace metals in the fermentor 9-fold during active growth, the biomass concentration was increased from 0.5 to about 1 g/L, and the biosorptive yield was increased from 130 µmol/L to 450 µmol/L when the experiment ended. There was some evidence of toxic effects of the minerals solution because growth was slower in the augmented medium compared to growth in the medium to which no supplementary trace metals were added.

Trace metals were added to a fermentor culture in the stationary phase, 60 h after inoculation (Figure 3.8), and after a further 3 h the mycelia showed signs of recommencement of growth (appearance of thin hyphal stubs on old, thickened hypha). Copper uptake by the biosorbent was improved by 30% at 65 h, but dropped again after 68 h, presumably when growth ceased. This indicates that the living biomass may be "activated" to increase subsequent metal uptake by the biosorbent.

Figure 3.6: Biosorbent Production in Shake Flasks During Additions of Trace Metals

○ without additions of trace metals

Δ trace metals increased by 100% of their initial concentration at times indicated with arrows

Medium B was used with two times the initial concentration of metals

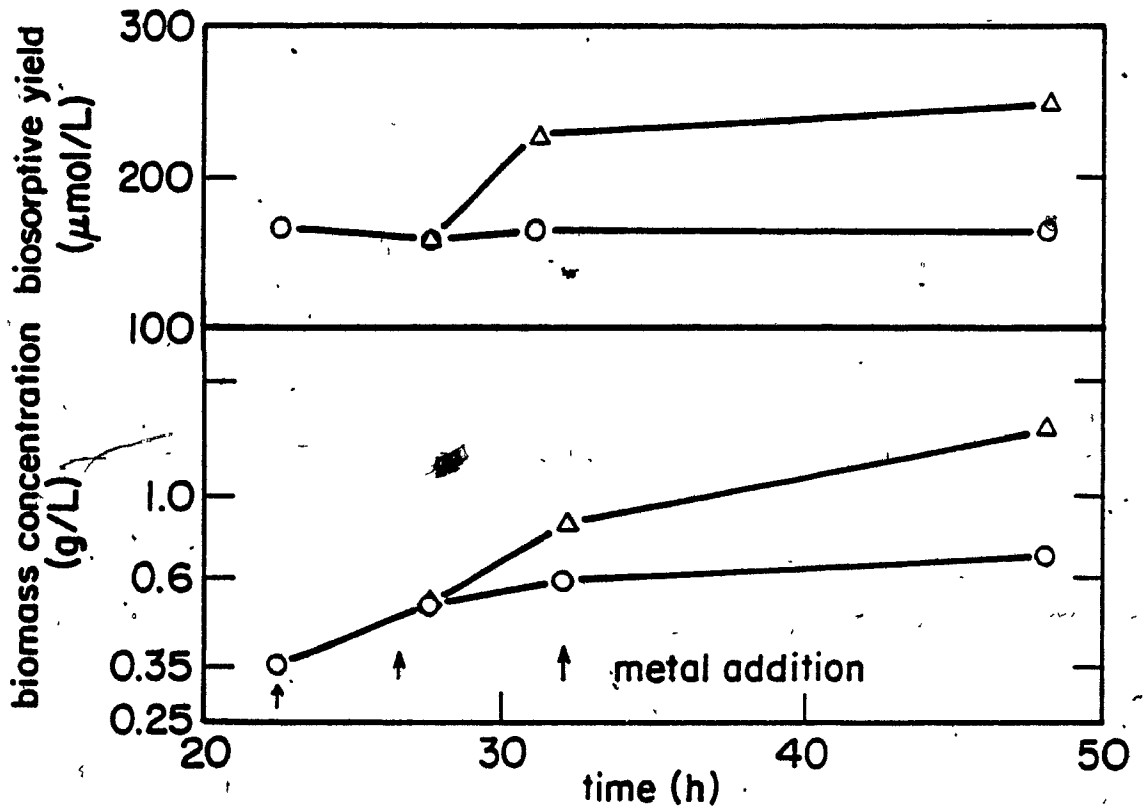


Figure 3.7: Biosorbent Production in Fermentors During Additions of Trace Metals

Δ without additions of trace metals

○ trace metals increased by 100% of their initial concentration at times indicated with arrows

Medium B was used with two times the initial concentration of metals

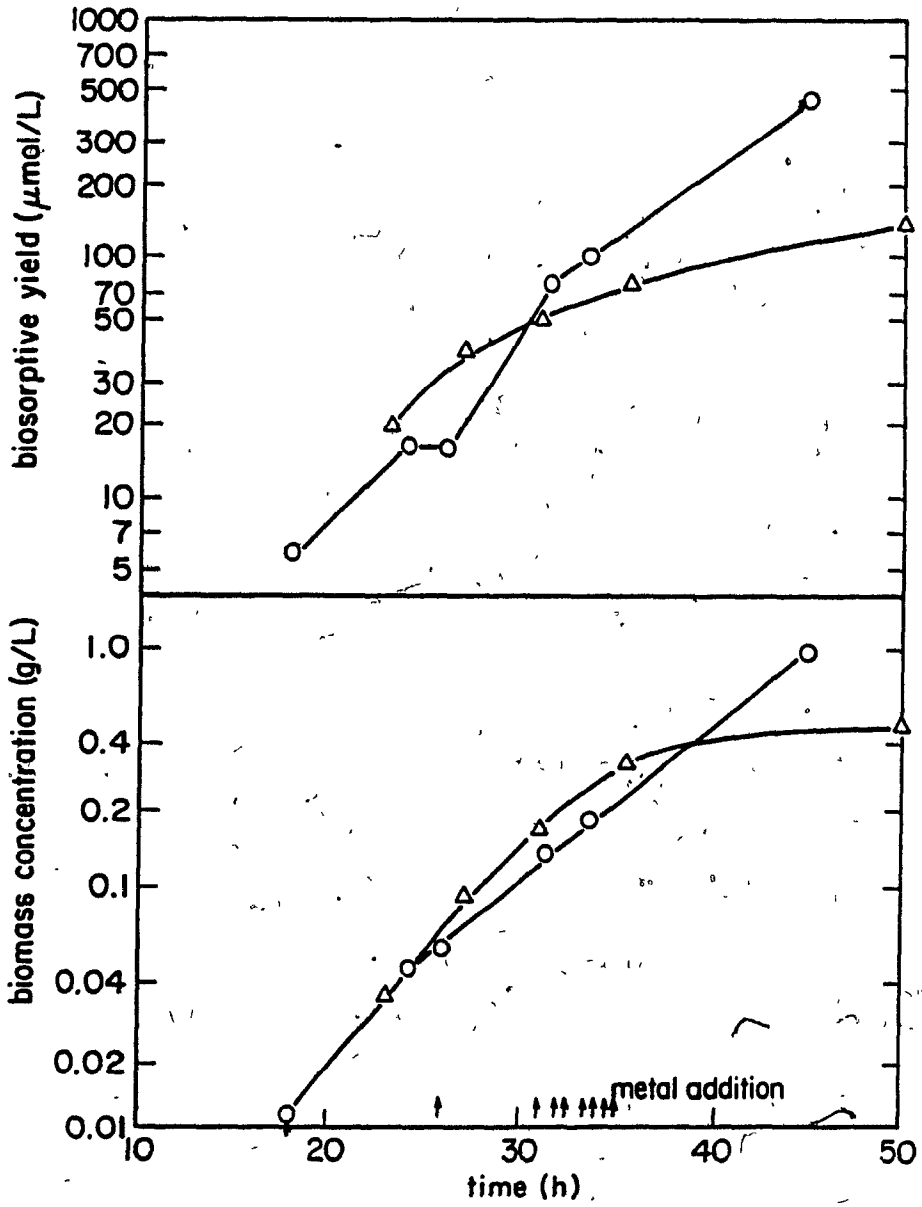
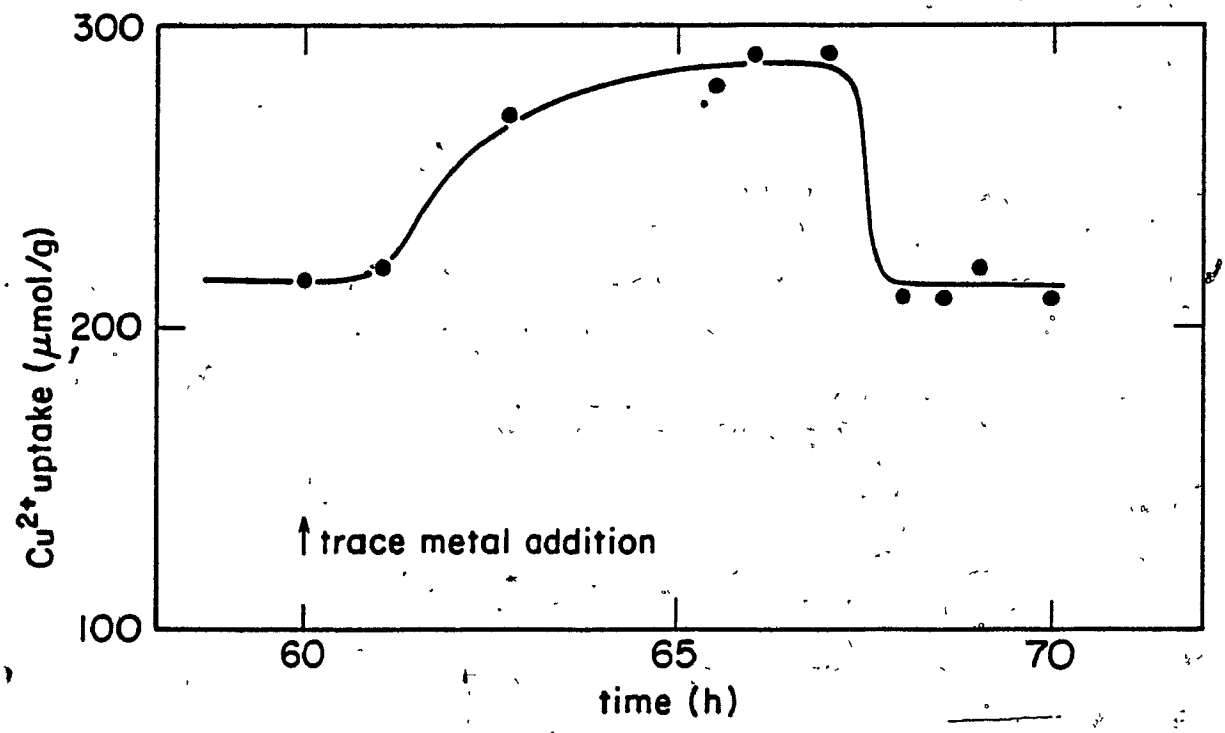


Figure 3.8: Enhancement of Cu-sequestering Ability of Old Biomass Grown in Medium B

Trace metals were added at five times their initial level at the time indicated by the arrow.

This experiment was performed twice; alternate points are the results of one of the replicas.



3.2.4.2 Toxicity experiments

Although adding a mixture of trace metals to the culture demonstrated that at least one essential element was being depleted, the possibility existed that others were toxic. Essentiality and toxicity of the salts in the preliminary media were investigated by augmenting an even more minimal medium (medium C; Table 2.2). Very low salts concentrations were used to minimize the possibility of toxic concentrations in the base-line medium. In a shake-flask experiment, a 20-h-old vegetative inoculum propagated in medium C, rather than a spore inoculum, was used to mimic the previous experiments in which metals were added after growth had commenced. Biomass concentration was measured after another 20 h, at which time the pH started to drop in the flasks with the highest biomass concentrations, but the glucose was not entirely depleted (Figure 3.9). Copper was the most toxic of the trace metals as it totally inhibited growth at a concentration of 4 μM, followed by nickel and cobalt which inhibited growth at a concentration of 40 μM. Manganese and molybdenum resulted in slightly lower biomass concentrations at all levels, whereas zinc had little or no effect. Chromium was stimulatory at a 0.5 μM concentration but slightly toxic at higher levels. Among the salts added in larger quantities, 5.3 mM $CaCl_2$ and 10 mM $MgSO_4$ stimulated growth slightly, and 40 mM KH_2PO_4 and 0.53 mM $FeCl_3$ enhanced growth markedly. At concentrations of 20 and 200 mM, NaCl resulted in lower biomass concentrations, whereas 2 M NaCl was extremely toxic. This salt was investigated thinking that other essential elements might be displaced from the biomass by NaCl, but stimulation of growth was not observed.

Figure 3.9: Effect of Different Levels of Selected Salts on the Biomass Concentration after 20 h of Propagation in Augmented Medium C

The control range represents the growth in the unaugmented medium.

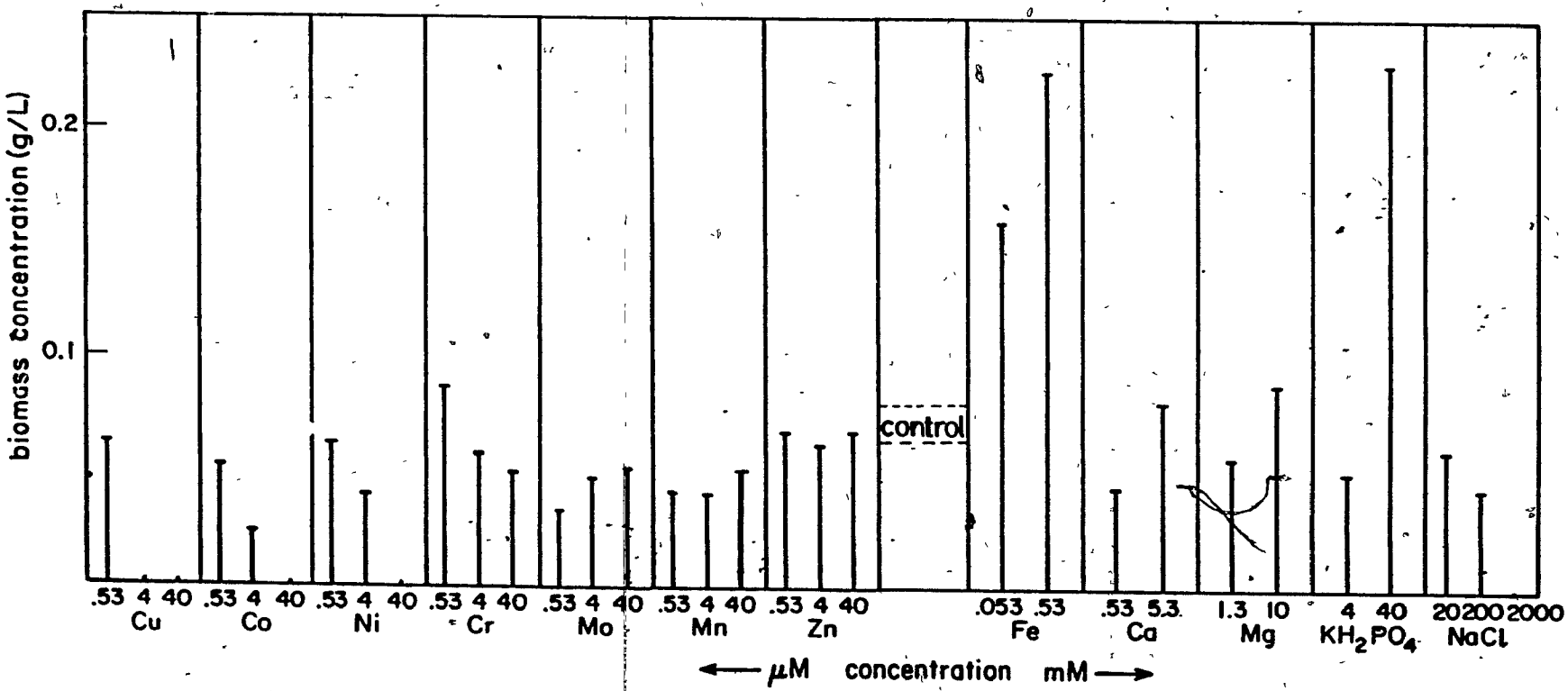


Table 3.10

R. javanicus Biomass Production in High Salts Media

Salt Concentration in Medium			Biomass Concentration (g/L)
Metals	KH_2PO_4	$MgSO_4$	
Hi	Hi	Hi	3.5
Hi	Hi	Lo	3.2
Hi	Lo	Hi	3.2
Hi	Lo	Lo	3.6
Lo	Hi	Hi	1.1
Lo	Hi	Lo	1.0
Lo	Lo	Hi	0.53
Lo	Lo	Lo	0.60

Concentration designations are:

metals -- Hi = $FeCl_3$, 400 μM; $ZnSO_4$, 40 μM; $MnSO_4$, 40 μM;
Lo = one tenth of the high levels

KH_2PO_4 -- Hi = 5 g/L; Lo = 1 g/L

$MgSO_4$ -- Hi = 1 g/L; Lo = 0.2 g/L

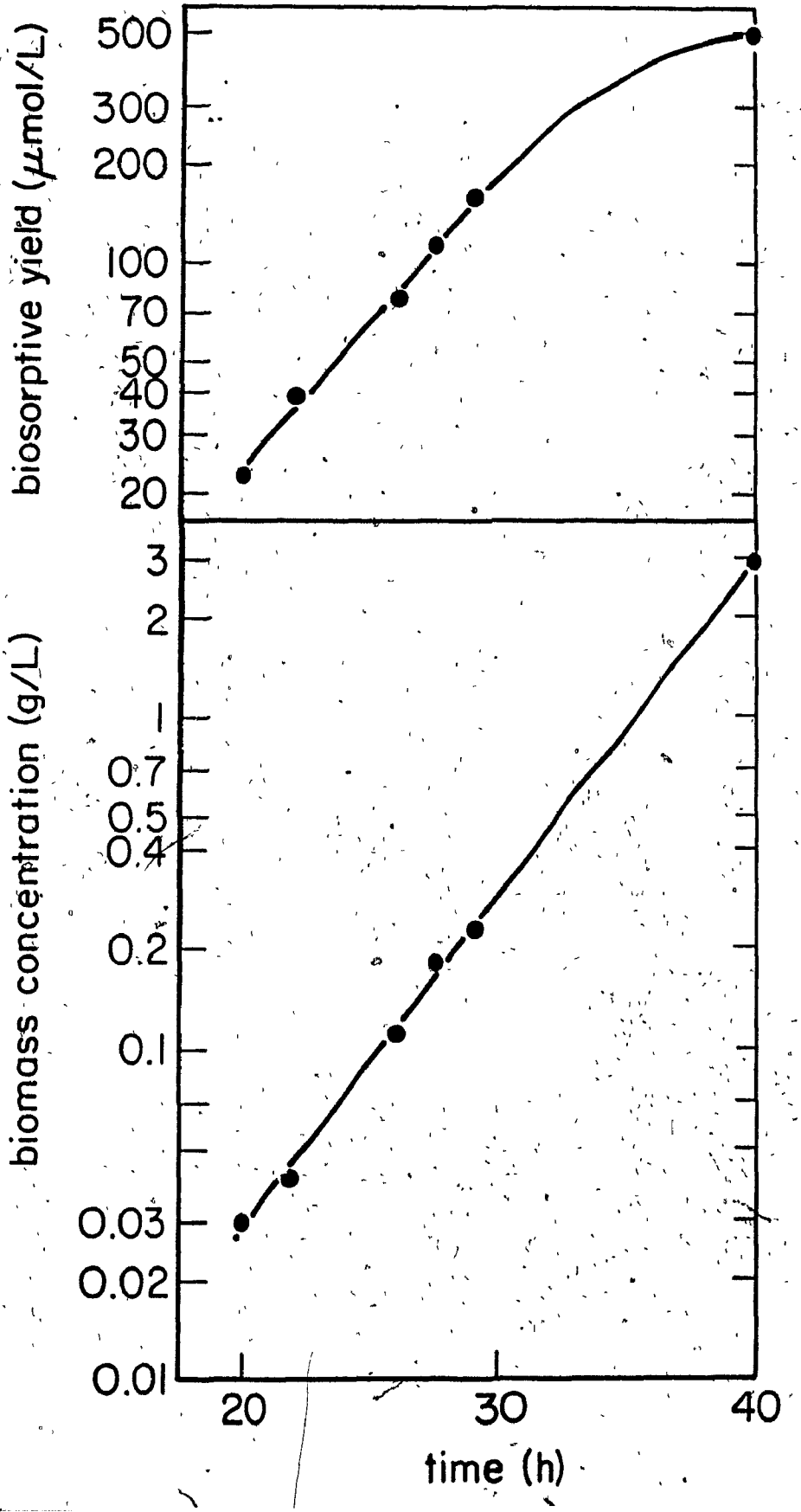
3.2.4.3 Growth of *R. javanicus* in the high salts medium

Based on the above findings, the high salts medium (HSM, Table 2.2) was devised. Glucose and urea concentrations were set at 20 and 4 g/L respectively. Calcium was added at a relatively high concentration (9 mM), and molybdenum and chromium were added at a 0.5 µM concentration. Copper, cobalt and nickel were added in low concentration (0.04 µM) because they are considered essential (Bowen, 1979), and yet were found to be toxic.

Before settling on the final medium composition, synergism among the remaining ingredients was checked in a three-squared experiment with 50 mL of medium in Erlenmeyer flasks, harvested shortly after the commencement of acid production (Table 3.10). The levels of potassium phosphate and magnesium sulphate were two of the variables, whereas the metals iron, manganese and zinc were grouped as the third. With the high metal concentrations, biomass concentrations of about 3.4 g/L were attained, regardless of the levels of the other salts. At low metal concentrations, a biomass concentration of 1 g/L was obtained with a high level of KH_2PO_4 (5 g/L), compared with a concentration of 0.6 g/L biomass with less KH_2PO_4 (1 g/L). Magnesium sulphate levels did not affect the biomass concentration. Thus, high levels of all components were included in the final HSM.

Figure 3.10: Propagation of R. javanicus Biosorbent in the High-Salts Medium

This experiment was performed twice, and individual points are from one of the two replicas.



Growth and biosorptive yield were followed in the HSM in Versatec fermentors (Figure 3.10). The specific Cu^{2+} uptake decreased from about 900 to 300 µmol/g while the biosorptive yield increased to 500 µmol/L as the biomass concentration surpassed 3 g/L at a growth rate of 0.23 h^{-1}. Although a precipitate was present initially, it was not evident microscopically at 20 h and after.

3.3 Effects of Biomass Age and Divalent Cations in the Growth Medium on Biosorbent Quality

Once the problem of propagation of biosorbent in quantity had been satisfactorily addressed, the propagation of biosorbents with different metal uptake characteristics was investigated. In this section of work, the previous observation that copper uptake behaviour varied with the time of harvesting (Tables 3.6 and 3.8) was investigated further. It was then hypothesized that this was at least in part due to fluctuations in the medium composition during batch growth, and investigations into the effects of divalent cations in the growth medium on biosorbent quality illustrated how to propagate a biosorbent with metal uptake characteristics which were different from that grown in the original HSM.

3.3.1 Variation of subsequent metal uptake behaviour with culture age

Eight Rhizopus species were screened for shifts in metal uptake behaviour with the time the organism spent in culture. In preliminary experiments R. chinensis and R. formosaensis grew very poorly on HSM solidified with 2% agar, but all grew well on the screening medium (SM, Table 2.2) with reduced calcium, iron,

Table 3.11

Screening of *Rhizopus* Species for Variation of Cu^{2+} Binding Strength with Biosorbent "Age"

time of culturing	18 h			22 h		
	Biomass (g/L)	W (μmol/g)	k (l/mM)	Biomass (g/L)	W (μmol/g)	k (l/mM)
R. arrhizus Fisher NRCCC# 2828	3.3[1]	36	7.2	3.8[2]	51	2.8
R. chinensis Saito ATCC# 22958 > NRCCC# 2834	3.3	240	0.3	4.0[1]	91	1
R. delemar var. *multiplici-sporus* Inui *et al.* ATCC# 24864 > NRCCC# 2829	3.8[1]	93	0.5	3.9[2]	50	2.5
R. formosaensis Nakazawa ATCC# 26612 > NRCCC# 2835	2.4	323	0.13	3.8	72	1.4
R. japonicus Vuillemin ATCC# 24863 > NRCCC# 2836	4.2[1]	110	0.4	5.4[1]	96	0.3
R. javanicus Takeda ATCC# 22580 > NRCCC# 2830	3.9[1]	800	0.06	4.0[2]	92.3	0.76
R. oligosporus Saito ATCC# 22959 > NRCCC# 2831	2.5	350	0.21	3.7	60	1.6
R. oryzea Went *et* Prinsen-Geerlings ATCCC# 12883 > NRCCC# 2832	1.8	132	0.56	2.5	40	5.3

1. glucose utilized; pH 3-6
2. glucose utilized; pH 6-8

Otherwise glucose remained in the growth medium and the pH was less than 6

The initial pH was 6.

manganese and zinc concentrations. Presumably one or more of these elements were at toxic levels in the HSM. The moulds were propagated for 18 h and 22 h from similar inoculum levels (1.5 - 4.5 x 10^9 spores/L) and the growth patterns and copper uptake behaviour are summarized in Table 3.11. The species were not all at the same stage of growth at the time of harvesting, as indicated by differences in the glucose levels and pH in the growth media. The pH of the growth media decreased initially, and then in some cases rose after the glucose was depleted and primary metabolites were oxidized. Many of the organisms formed large pellets in the flasks, although disperse growth was observed with R. chinensis, R. delemar var. multiplici-sporus, R. japonicus and R. javanicus.

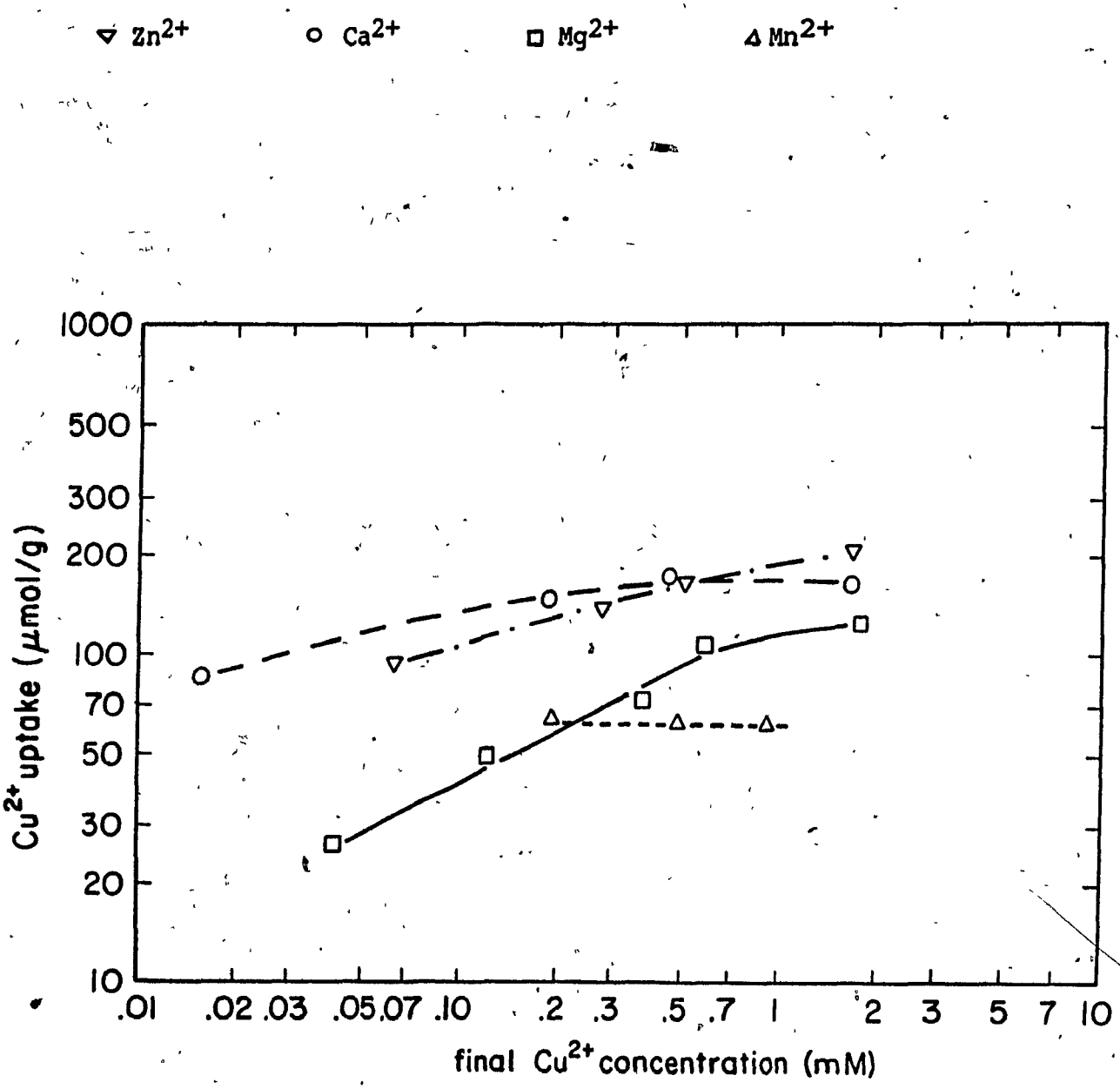
Copper binding strength (apparent equilibrium constant, k) and total numbers of sites on the biosorbents (specific uptake at saturation, W) were calculated from four determinations at final solution concentrations between 0.5 and 1 mM using the equilibrium model (Section 3.2). This was in fact a linearized form of Langmuir's equation (inverse uptake versus inverse solution metal concentration). The R. arrhizus samples exhibited very low uptake values which were accurate to within 10% due to the small differences between initial and final solution metal concentrations, and this is not sufficient accuracy to draw conclusions about the relative magnitudes of the W and k values. Otherwise, the concentration of sites was similar or lower on "older" biosorbents compared to the "younger" ones, and these sites were either of the same strength or stronger on the "old" biosorbents compared to the "younger" ones.

3.3.2 Effects of divalent cations in the growth medium on subsequent copper uptake

In all of these studies the moulds have been grown in batch culture. This means that there is a strong possibility that the "aging" of the mycelium was due to changes in the levels of nutrients as they were utilized. The effects of fluctuations of concentrations of medium components may be eliminated by growing the organism in continuous culture, which is very difficult with this mould. Failing that, these effects may be minimized by propagating the mycelium to low final biomass concentrations in media in which components are severely limited or in excess.

In light of previous experience that the cation content of the medium was important, R. javanicus was propagated in shake flasks (100 mL medium) in four media based on a minimal medium (MM, Table 2.2) which was supplemented with one of either manganese (II), zinc, calcium or magnesium at ten times the level of the other three metals. The basal concentration of these cations (4 μM) was low compared to previous media in order to highlight the role of the dominant cation. The mould was grown for 44 hours from an inoculum of 9×10^8 spores/L and the Cu^{2+} uptake of the resulting biosorbents was measured (Figure 3.11). Poor growth was observed in the high-Mn medium; the pH rose to 8, the flasks smelled of ammonia, and only 0.13 grams of biosorbent per litre of medium was produced. This biosorbent was saturated at 0.2 mM Cu^{2+}, with a total of 60 μmol Cu^{2+} bound per gram of biosorbent. In the high-Zn medium growth was somewhat better (0.18 g/L), and the biosorbent had an apparent equilibrium constant of 16 mM^{-1} and a total of 220 μmol sites/g biomass.

Figure 3.11: Cu^{2+} Uptake by R. javanicus Biosorbents Propagated in the Minimal Medium Supplemented with Zn^{2+}, Ca^{2+}, Mg^{2+}, or Mn^{2+}



The organism grew even better in the high-Ca medium (0.22 g/L), and the biosorbent exhibited copper uptake similar to the sample from the high-Zn medium over the concentration range examined, although the binding was stronger (apparent equilibrium constant was 62 mM^{-1}) and the total number of sites was lower (160 µmol sites/g biomass. The highest biomass concentration (0.39 g/L) was attained in the high-Mg medium, but this biosorbent had an apparent equilibrium constant of only 10 mM^{-1} and 110 µmol sites/g biosorbent.

The question still remained whether differences in the biosorbents were directly attributable to the designed predominance of cations in the original medium or whether, in cultures with varying biomass concentrations, depletion of another medium component led indirectly to the differences. The best biomass production and the largest differences in binding constants were attained with either calcium or magnesium as the predominant cation, so this result was pursued using the SM with either 4 µM or 40 µM of these ions. The organism was grown under conditions which were similar to those of the previous experiment; it was propagated for 46 h from an inoculum concentration of 3×10^8 spores/L in 100 mL of medium and final medium pH values were 7.1-7.2 for the high-Mg cultures and 6.3-6.6 for the low-Mg ones. There was a marked difference in the morphology of the cultures grown with high calcium levels, compared to the low-calcium cultures. Under microscopic examination the hyphae were about 50% larger in diameter in the high-calcium medium.

Table 3.12

Properties of R. javanicus Biosorbents Propagated in the Screening Medium with Various Concentrations of Magnesium and Calcium

Concentration			Uptake Parameters	
Mg^{2+} (μM)	Ca^{2+} (μM)	Biomass (g/L)	W (μmol/g)	k (mM^{-1})
40	40	1.2	400	3
40	4	0.8	290	7
4	40	0.35	250	0.8
4	4	0.25	270	16

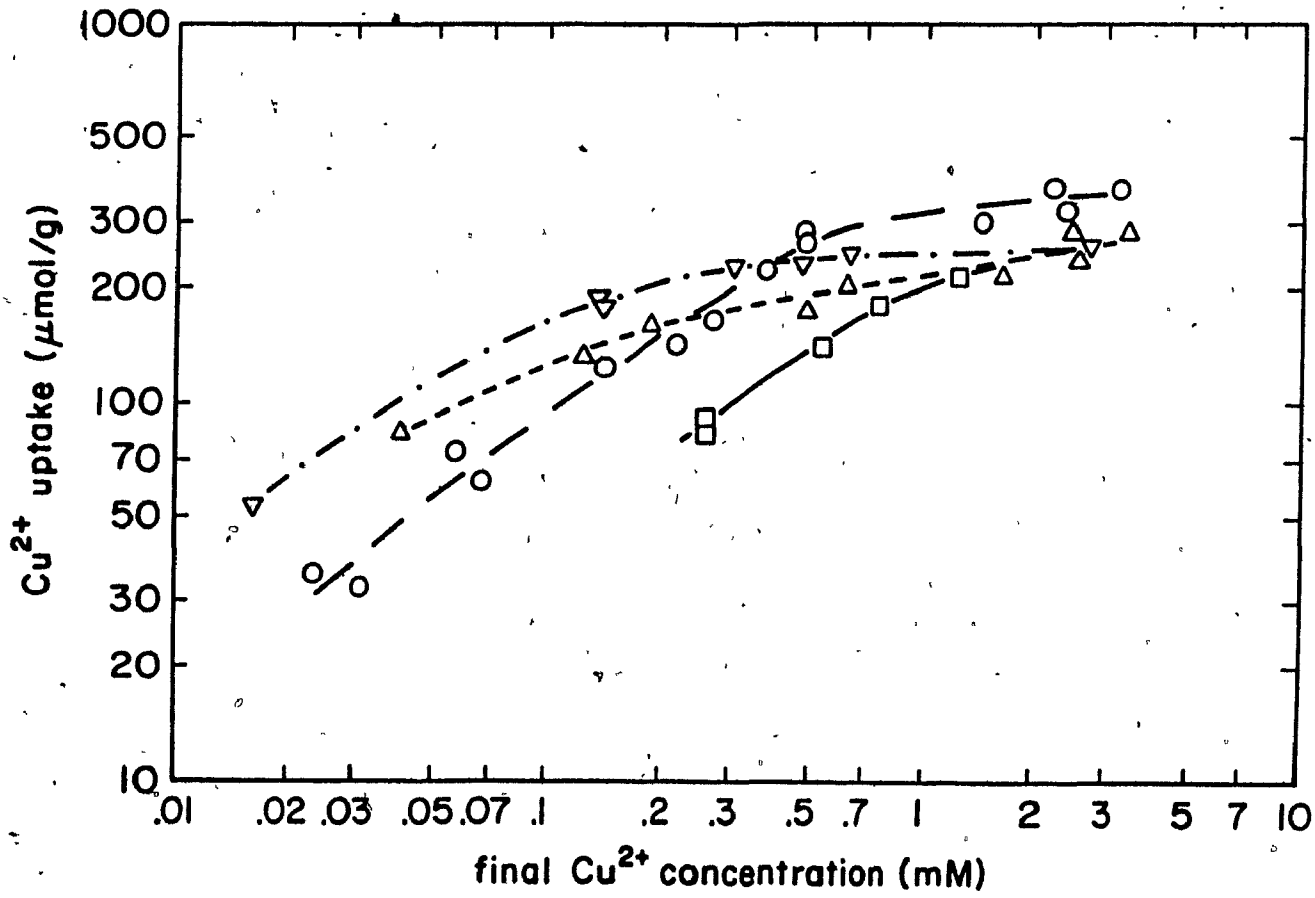
Biosorbent production and characteristics are summarized in Table 3.12 and the copper-uptake curves are presented as Figure 3.12. High levels of magnesium in the growth medium resulted in an increased biosorptive yield, principally because biomass concentrations in the growth media were higher. Biosorbents from growth media with low calcium levels exhibited much stronger binding of the cupric ion than did biosorbents from high-Ca media. The observed role of calcium in determining the apparent strength of binding was not due to fluctuations of other medium components brought about by variations in the biomass concentration in the growth medium. There was no correlation between the amount of growth (biomass concentration in the growth medium) and the apparent strength of binding.

Figure 3.12: Cu^{2+} Uptake by R. javanicus Biosorbents Propagated in the SM with 4 µM (L) or 40 µM (H) Magnesium and Calcium

○ H Mg, H Ca △ H Mg, L Ca

□ L Mg, H Ca ▽ L Mg, L Ca

This experiment was performed twice, and individual points are the results of one of the replicas



3.4 Extraction and Characterization of R. javanicus Biosorbents Propagated in Two Media

In the previous section it was discovered that the biosorbent characteristics could be affected by both the growth time and the medium composition. With this knowledge it was possible to grow samples to be used in the final section of this work, in which differences in the chemistry, ion exchange and metal uptake behaviour of R. javanicus biosorbents which had been grown under three different conditions were analysed. Samples of each of the three biomass types were subjected to an acid-wash similar to the standard treatment used in the previous studies, as well as to mild extractions at room temperature with lithium chloride and nitrous acid to remove polar polymers with minimal disruption of the rest of the biosorbent. The chemical composition of the nine resulting biosorbents was determined, and the ion-exchange behaviour of one set of three samples (standard plus two extracted biosorbents derived from a single biomass sample) was examined by titration with sodium hydroxide. Uptake of manganese (II), chromium (III) and copper (II) by all of the biosorbents was measured to investigate the possibility of variations in the relative strength of binding with changing culture conditions. The concentration range over which metal uptake was measured was extended to cover four orders of magnitude, to allow calculation of strength of binding of more than one apparent binding site, should such a model be appropriate.

3.4.1 Propagation, extraction and chemical analysis of biosorbents

Three large batches of Rhizopus javanicus for detailed analysis of the biosorbent chemistry and metal uptake behaviour were propagated in New Brunswick fermentors (14 L Microferm; 10 L nutrient broth). Two lots were grown for different periods of time (26 h and 45 h) in the high-salts medium (HSM) and one batch of the mould was grown to a low final biomass concentration in a variation of the HSM with higher levels of calcium and zinc and lower levels of magnesium (HCZ, Table 2.2). Morphological differences between the HSM and HCZ biosorbents were observed as the hyphae grown in the higher calcium concentration were about 50% larger in diameter. In this section of work the acid wash was not performed initially; repeated water-washes and resuspension in a Waring blender were carried out until a clear, colourless filtrate was obtained for several rinses. This entailed more than ten washes. As was observed previously (Section 3.1) the preliminary water-washing and suspending in a Waring blender resulted in a large proportion of hyphal "ghosts", leaving only some tips intact where they were separated from the larger mass by septa. The drying and grinding procedures caused disruption of the intact segments, and the final washed or extracted materials were microscopically free of cytoplasm (only sheets of what appeared to be debris were observed) and had undergone losses of 12-72% of the weight of the partially purified water-washed samples (Table 3.13). Differences in the structure of the biomass samples were immediately evident as the HCZ biomass was the most susceptible to extraction, while the young HSM biomass was the most refractory.

Table 3.13

Extractions and Chemical Analysis of Biosorbents

Sample*	Fraction not Extracted**	PO_4 (μmol/g)	Uronic Acid (μmol/g)	Glucosamine (μmol/g)	Protein (g/g)
Young HSM					
AW	(0.88)	1240	1390	1700	0.18
LE	0.87	830	1320	1930	0.20
NE	0.60	300	1200	2860	0.24
Old HSM					
AW	(0.71)	1130	1270	1620	0.14
LE	0.77	830	1710	2140	0.17
NE	0.45	180	1650	3270	0.24
HCZ					
AW	(0.57)	810	1450	1480	0.43
LE	0.69	710	1470	2180	0.16
NE	0.49	29	1000	2420	0.19

* AW = acid-washed; LE = LiCl-extracted; NE = HNO_2-extracted

** Figures in brackets are the mass fraction of the water-washed samples which were not extracted by the acid wash; other results are on the basis of the acid-washed samples.

The water-washed, dried and ground biomass samples were washed with 0.01 M HCl (standard treatment) or extracted with 5 M LiCl or 3 M HNO_2 solutions. The results of the extractions and chemical assays are presented in Table 3.13. Lithium chloride extracted about 40% of the phosphate from all the samples, as well as varying quantities of uronic acid, chitosan and protein. Nitrous acid removed most of the phosphate, all of the unacetylated glucosamine residues and varying quantities of uronic acid and protein, leaving material that was in all cases enriched with glucosamine.

The phosphate levels in the biosorbents were quite high (800 - 1200 umol/g). As well, the phosphate was labile and was totally dissolved by the mild hydrolysis procedure (2 h at 100°C in 1.2 M HCl) as well as by the harsh treatment (10 h at 100°C in 6 M HCl) (Appendix 1). Therefore both the mild and harsh hydrolysates were analysed for phosphate. The results agreed within 2%, which indicated that no major losses occurred during the hydrolysis procedures.

Neutral sugars (glucose equivalent) as measured by the anthrone method were in all cases less than 15% of the uronic acid levels, and at this level may be attributable to interferences from proteins and uronic acids (Shields and Burnett, 1960). For this reason neutral sugars were disregarded in the final compilation of the data. Nevertheless, there were quite probably small quantities of them in the samples, as variable but small amounts of mannose, fucose, and galactose have been reported in cell walls of mucoralean fungi (Bartinicki-Garcia and Reyes, 1968a, Datema *et al.*, 1977a, and Dow *et al.*

1983). Glucuronic acid was the only acidic sugar found in the samples described in the literature, and this was assumed to be the case here as well.

Glucosamine was measured directly by HPLC, and those determinations agreed within 20% with the difference between the ninhydrin measurement of protein plus glucosamine and the Lowry measurement of protein. The accuracy of the protein measurement cannot be assessed because the standard was not necessarily appropriate (Lowry et al., 1951). The degree of acetylation of the glucosamine was assessed by high-performance liquid chromatographic analysis of the nitrous acid degradation products of unacetylated glucosamine, and analysis of the glucosamine in the mild acid hydrolysates. Chitin-plus-chitosan content of the acid-washed samples ranged from 37% in the young biosorbent grown in the HSM to 28% in the old biosorbent. The degree of acetylation of these polymers was radically different as nitrous acid reacted with only 10% of the glucosamine in the young HSM biosorbent, 30% in the HCZ biosorbent and 55% in the old HSM biosorbent. Nitrous acid most probably reacted with not only glucosamine, but other components of the biosorbent as well, such as proteins, to produce nitrated coumpounds and nitrosamines (Noller, 1965). Contamination with such highly-coloured compounds would account for the slightly darker, orange-brown colour of these extracted materials compared to the other pale biosorbents.

Both the extracts and solids were analysed for phosphate, neutral sugars, uronic acids, glucosamine and protein, and mass balances among the extract, biosorbent and water-washed biomass

sample assays were generally within 10%, although high chloride or nitrous acid levels interfered with some assays of the extracts (Section 2.4). The materials detected account for 85-100% of the mass of the samples, which may be considered virtually all of the mass because of the uncertainty associated with the protein assays.

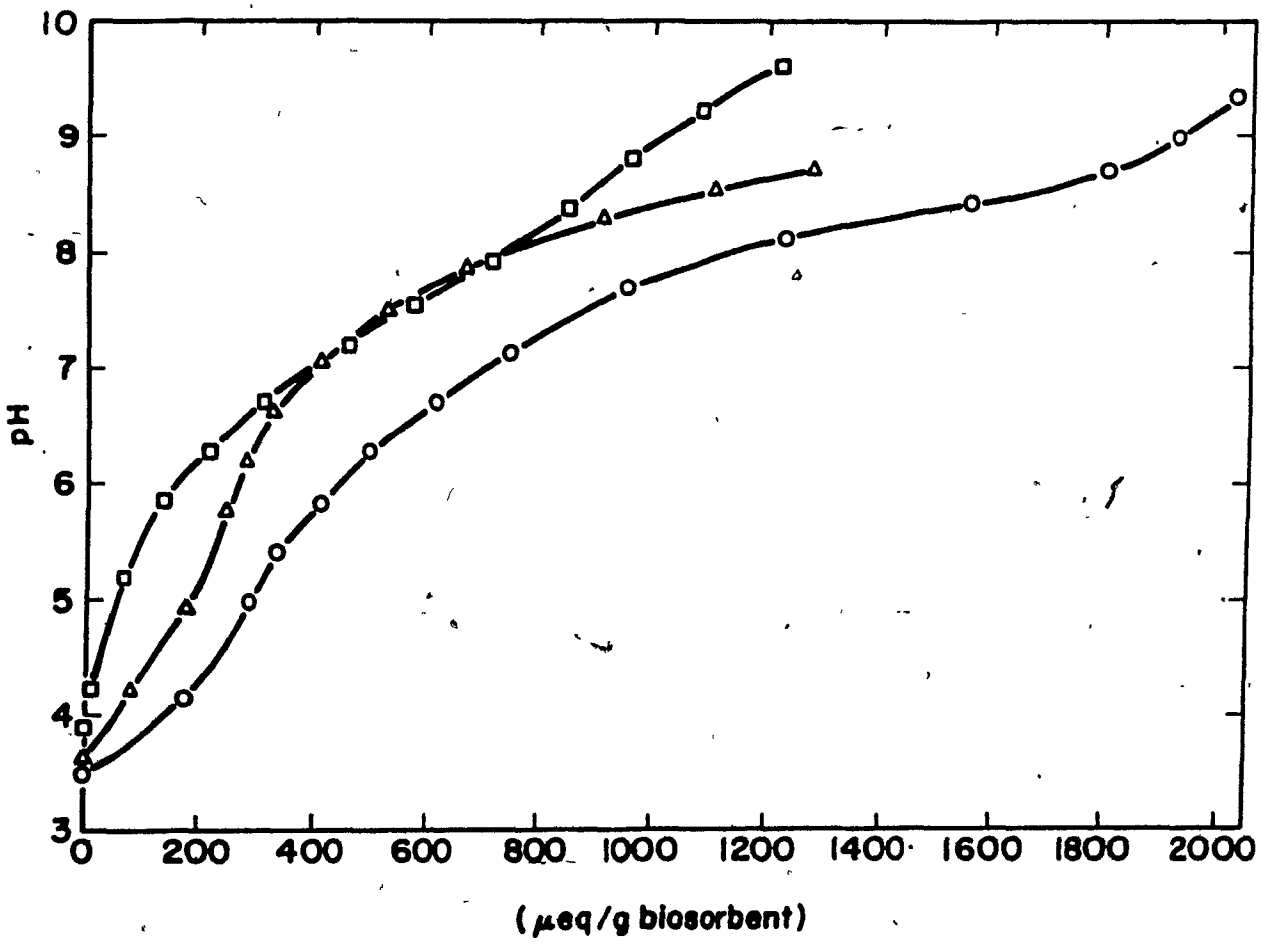
3.4.2 Alkali titrations of old biosorbents propagated in the high salts medium

Finely ground samples of acid-washed and extracted old HSM biosorbents were titrated slowly under nitrogen in aqueous suspension with sodium hydroxide and the curves are presented in Figure 3.13. The acid-washed biosorbent had the highest overall buffering or ion exchange capacity, although in all cases about half of the oxygen-containing ionizable groups (phosphate and uronic acid) present according to the chemical analyses were available for ion exchange. The nitrous acid-extracted sample had the lowest buffering capacity, reflecting the removal of ionizable polymers from the sample. In particular, the absence of buffering by the nitrous acid-extracted biosorbent at higher pH values (>8) corresponded to the removal of free amine groups.

During these titrations the pH measurements were accurate to within 0.05 units, and the alkali consumption was accurate to within 1%.

Figure 3.13: Titration of R. javanicus Biosorbent Slurries with NaOH

o Acid-washed Δ LiCl-extracted □ HNO_2-extracted



3.4.3 Cu^{2+}, Mn^{2+} and Cr^{3+} uptake by the biosorbents

Uptake of copper(II), manganese(II) and chromium(III) by all nine biosorbent samples was measured at metal concentrations between 1 µM and 10^4 µM and the curves are presented as Figures 3.14, 3.15 and 3.16. The acid-washed HSM biosorbents exhibited behaviour which was similar to one another, with copper and manganese uptake curves superimposed and much lower uptake of chromium (Figures 3.14a and 3.15a), whereas the HCZ biosorbent exhibited lower binding of manganese than copper at a given solution concentration (Figure 3.16a). All extracted samples had a lower affinity for manganese than copper, and compared to the rest of the biosorbents, the nitrous acid-treated samples exhibited much stronger binding of all the metals, although the capacity was lower.

Fitting of the data to a multiple-site equilibrium model was carried out (Table 3.14). It must be noted that the equilibrium constants and concentrations of sites were heavily coupled and this data is of empirical value only. A single-site model was in most cases inappropriate over the entire range of metal concentrations examined, but the introduction of a second type of uptake site to the model resulted in good fits. This summary of the uptake behaviour is useful for comparison of various samples, and makes clear that the uptake capacity of the biosorbents, as well as the relative strengths of binding of several metals, may be influenced by the conditions of propagation and the treatment of the biomass.

Figure 3.14: Metal Uptake by R. javanicus Propagated for 26 h in the High Salts Medium (Young HSM Biosorbents)

a) acid-washed b) LiCl-extracted c) HNO_2-extracted

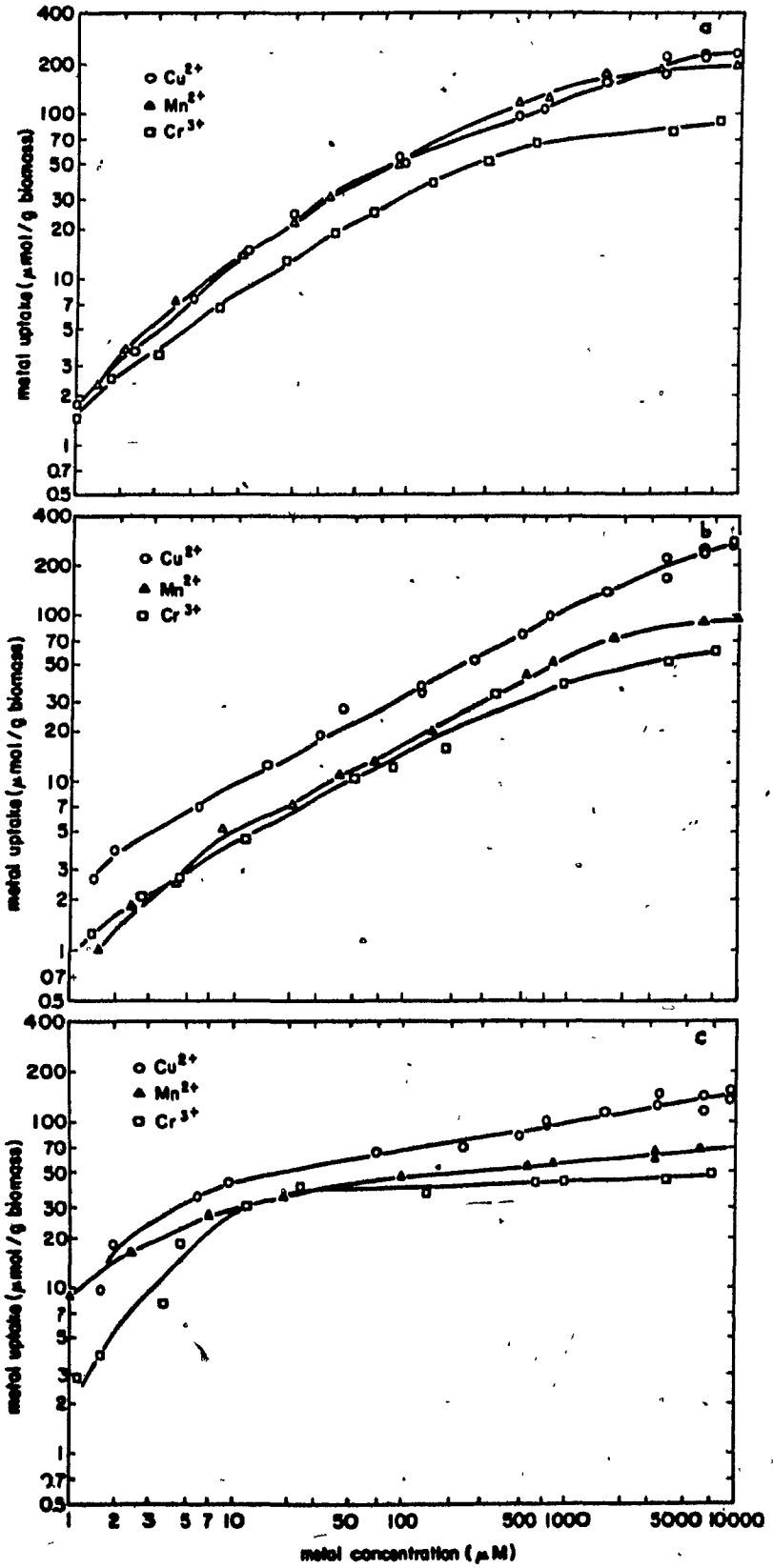


Figure 3.15: Metal Uptake by R. javanicus Propagated for 45 h in the High Salts Medium (Old HSM Biosorbents)

a) acid-washed b) LiCl-extracted c) HNO_2-extracted

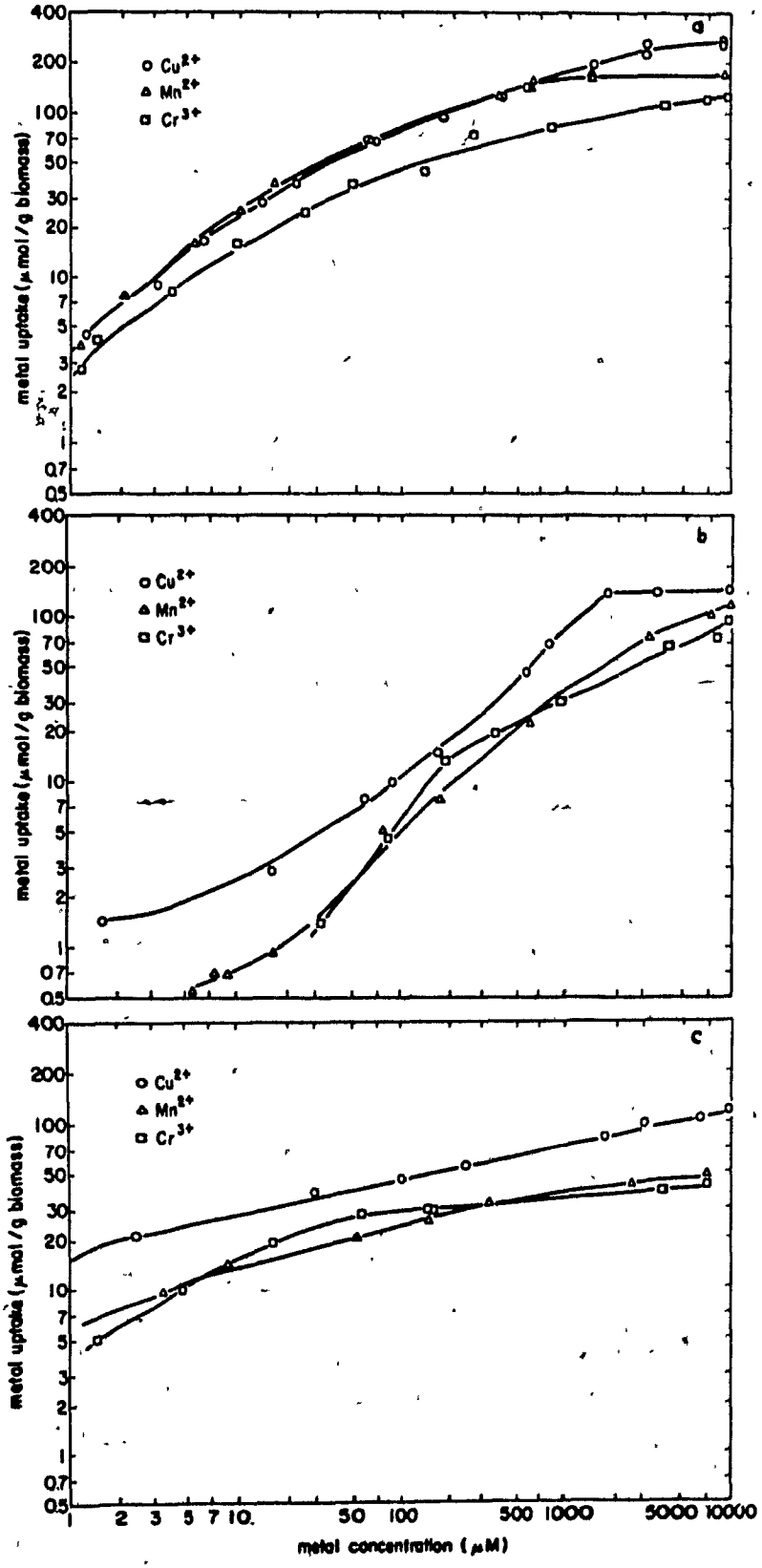


Figure 3.16: Metal Uptake by R. javanicus Propagated for 35 h in the High Calcium and Zinc Medium (HCZ Biosorbents)

a) acid-washed b) LiCl-extracted c) HNO_2-extracted

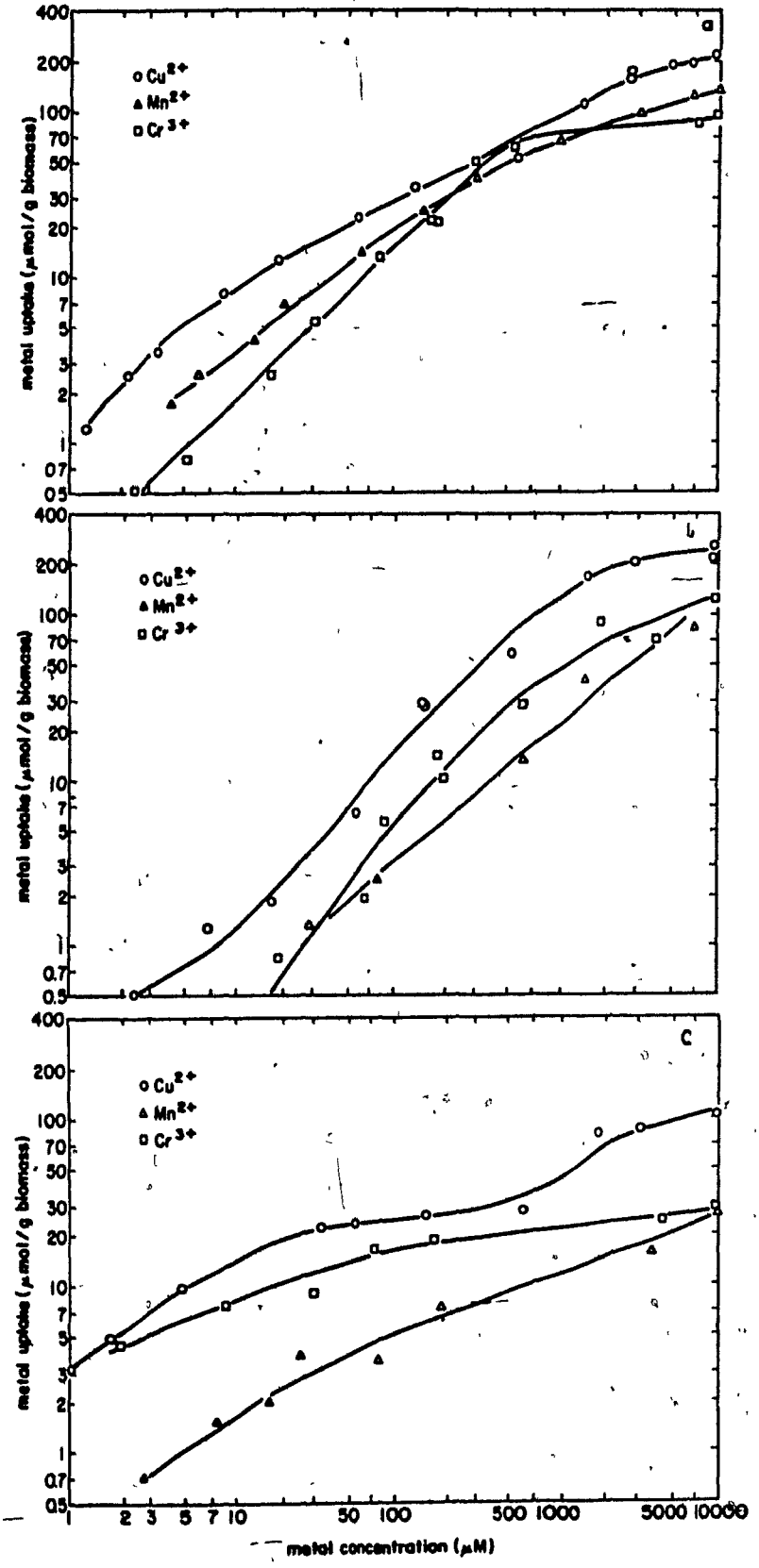


Table 3.14
Apparent Equilibrium Constants and Uptake Site Concentrations for R. javanicus Biosorbents

Sample*	Cu^{2+} k	Cu^{2+} W	Mn^{2+} k	Mn^{2+} W	Cr^{3+} k	Cr^{3+} W
Young HSM						
AW	23	67	14	120	84	14
	0.31	260	**	**	2.5	91
LE	160	15	90	7.9	180	5.3
	0.76	240	0.94	96	1.6	70
NE	120	70	340	38	32	51
	0.41	110	0.98	41	**	**
Old HSM						
AW	69	50	49	67	65	40
	1.2	240	2.1	130	0.83	96
LE	0.86	170	0.43	140	0.53	103
	**	**	**	**	**	**
NE	1400	28	850	14	130	31
	0.75	102	3.6	36	0.21	18
HCZ						
AW	39	27	42	8.3	1.1	110
	0.42	240	1*0	130	**	**
LE	0.37	390	0.01	1300	0.13	250
	**	**	**	**	**	**
NE	190	21	66	4.5	420	8.5
	0.51	110	0.11	46	4.5	19

* AW = acid-washed; LE = LiCl-extracted; NE = HNO_2-extracted

** Only one value was needed to model the metal uptake behaviour, or the 2-site model did not converge.

4. DISCUSSION

4.1 Propagation of Biosorbent

4.1.1 Inoculum preparation

Reproducibility of growth experiments involving any microorganism depends upon careful control not only of the period of growth during the experiment itself, but of all the steps leading up to it. This is particularily true in the case of the more complicated microbes such as moulds which commonly appear to have a "memory" of their past growth conditions which is much longer than could be explained by institution of new metabolic pathways, or by carry-over of traces of previous media in successive transfers. One obvious manifestation of this is the long period of adaptation needed before moulds may be reproducibly grown on any particular medium. In this work the most dramatic demonstration of the phenomenon occurred when transferring R. japonicus from solidified organic medium on which it sporulated only occasionally to the synthetic media which supported profuse formation of sporangia, or vice-versa. The fact that the periods for adaptation were the same for transfers between either medium indicates that dilution of a component of the previous medium was not responsible for the changes in culture morphology. To ensure that the morphology of

the mould was related as closely as possible to the medium composition, the standard procedure which was adopted for all growth experiments was that at least five successive transfers on slants of any particular solidified medium must precede preparation of spore inocula. The number five was chosen because after four transfers of R. japonicus as discussed above the morphology on subsequent transfers was consistent, and the fifth transfer on a slant as well as the subsequent transfer to a larger surface of solid medium were considered an adequate safety margin. This represented a month of growth on solid medium before growth in suspension (six transfers times five days per transfer). Application of this procedure demonstrated that any particular medium was complete (would support good growth) and resulted not only in repeatable harvesting of spores, but also in repeatable growth patterns and subsequent metal uptake behaviour. The overall experimental reproducibility was illustrated by several sets of results, such as those in Figures 3.3, 3.8, 3.10, 3.12 and Table 3.6, which show data from replicate experiments. During the course of other experiments which were performed only once, replicate samples were taken and analysed.

The multiple-transfer procedure which was adopted minimized the lag period in suspension culture (about half a day). Extended lag periods were commonly observed in the first stages of adaptation to any new medium. In other work not directly related to this (Treen-Sears, unpublished) a lag period of two to four days was observed before significant quantities of biomass were produced when Rhizopus species were transferred from the solidified organic medium to sterile whey, a radically different

growth medium, without the successive transfers to allow the organism to adapt. During the course of this work it was not practical to reduce the lag period by using a vigorous vegetative inoculum because aseptate hyphae which are not amenable to partial homogenization before dispensing are difficult to handle reproducibly. As well, attempts to grow a more easily manipulated yeast-like inoculum of R. javanicus under a variety of conditions reported to induce such growth in species of the closely-related genus, Mucor, were unsuccessful. The absence of yeast cells of several other Rhizopus sp. under anaerobic conditions which caused such growth in Mucor has been observed previously (Bartinicki-Garcia and Nickerson, 1962a; Hesseltine et al., 1985). In previous studies, however, the organisms did grow anaerobically. The absence of such growth by R. javanicus in this study may be a characteristic of this species, or may be due to increased nutritional demands under anaerobic conditions, as was observed for Mucor rouxii (Bartinicki-Garcia and Nickerson, 1962b; Elmer and Nickerson, 1970). When any microbe is being grown for profit, long delays due to extended lag phases must be avoided, as the maintenance of the unproductive organism in the growth medium wastes both energy and time in the fermentation vessel and increases the likelihood of contamination.

The reason for these organisms' "memory" of previous growth conditions is not entirely clear, although the genetic material is most probably involved. As reviewed by Burnett (1976, pp 480-481), Phycomycetes are heterokaryons (within one hypha there are many nuclei which do not all contain identical genetic material), and they grow from multinucleate sporangiospores which themselves

arise from the collection of nuclei rather than the splitting of one original nucleus. In Mucoraceae, which in pure culture do not undergo hyphal fusion, mutation and selection are the only means of evolution of the fungal morphology. Since the time scales normally involved in uninduced mutation are much greater than in these studies, the adoptation of a new morphology during successive transfers on a single medium following transfer from a different medium must be due to selection which led to a new stable distribution of the various types of nuclei. As long as no genetic material is actually eliminated in the process of such selection, reversion of the mould to its previous morphology and physiology upon transfer to the original medium would occur, as was observed in this work. The occurrence of such reversion indicates that mutation did not occur, and in general could be an indicator of strain stability, which is always a concern in industrial fermentations (Pirt, 1983). In further recognition of the possible variation among heterokaryotic species, recent investigations of the DNA complementarity of Rhizopus species has led to proposed simplifications of the taxonomy of the R. arrhizus-R. oryzea group as some species exhibited high relatedness to one another (J.J. Ellis, 1985).

Another implication of variations in fungal morphology on different growth media is that they may be valuable taxonomic indicators. Traditionally a variety of traits including the maximum temperature at which they will grow, metabolic characteristics and the morphology of reproductive structures have been used to delineate Rhizopus species (Inui et al., 1965; D.H. Ellis, 1981). However, some confusion has resulted from

difficulties in describing sporangial appearance unequivocally because of both strain variations and failure of some investigators to recognize the extreme effects which growth conditions may have on morphology (J.J. Ellis, 1981). "Synthetic Mucor Medium" (Hesseltine and Anderson, 1957) has been recommended for taxonomic studies of Mucorales (Hesseltine and Ellis, 1973). In fact, however, this is an incomplete medium. The essential trace minerals presumably were supplied by impurities in the chemicals or water, or during transfer from other maintenance media. The synthetic media developed in this work are the first defined ones for Rhizopus species which were proven over the course of several transfers to be complete. They would not be suitable for Mucor, however, because this genus requires vitamins which were not included. Variations of morphology during growth on different media are not common taxonomic indicators, no doubt because extreme and clear-cut variations such as that observed for R. japonicus are uncommon and purely serendipitous. Such phenomena may, however, provide a simple test for the species.

4.1.2 Pattern of growth of R. javanicus in suspension culture

All propagation of biosorbents was carried out in batch suspension culture, so a picture of the dynamics of growth is essential before addressing the effects of growth conditions on subsequent biosorbent quality.

A lag period of several hours preceded commencement of rapid growth (Section 4.1.1), but then the mould grew exponentially at specific growth rates which were observed in the Versatec fermentors to be from 0.08 h^{-1} to 0.24 h^{-1}, and in the high-salts

medium (HSM) exponential growth continued to a biomass concentration of 3 g/L (Figure 3.10). The fact that this type of growth was observed rather than linear growth, as is often the case with mycelial organisms which do not divide as do unicellular microbes, indicates that the organism was vigorous and that frequent hyphal branching occurred (Morrison and Righelato, 1974; Latge and Moletta, 1983). Clearly maximization of the rate of exponential growth would be desirable for industrial propagation of biosorbents, should all else be equal. However, biosorbent quality was also related to the rate of growth, and this is discussed in Section 4.1.4. The rate of exponential growth was affected by the medium pH (optimum pH=5), the presence of light and the level of carbon dioxide.

The fact that mucoraceous fungi are sensitive to light has long been recognized; effects of light include phototropism and the necessity of light (often periodic light and dark periods) for differentiation of sporangia. The rate of growth of the sporangiophore at different stages during development of the reproductive apparatus may also be affected by the presence or absence of light, and β-carotene production is stimulated and alcohol dehydrogenase production is inhibited by illumination of some organisms (Burnett, 1976; Bergman et al., 1969; reviewed by Senger, 1984). Chitin synthesis and breakdown have been implicated in the light growth response; 10%-30% stimulation of chitin synthetase upon illumination of crude extracts of Phycomyces blakesleeanus and 50% stimulation of the synthetase in vivo were reported (Herrera-Estrella and Ruiz-Herrera, 1983). In Aspergillus gigantus mut. alba, which is less closely related to

Rhizopus, the presence of light was found to stimulate chitin synthesis, and the chitin content of illuminated cultures was 50%-80% greater than those grown in the dark (Fiema and Globiewska, 1981; Fiema, 1983).

During the course of this work the specific growth rate in suspension culture was increased by 50% in the illuminated cultures compared to the ones in the dark, and the metal uptake behaviour was also affected. This is remarkably similar to the stimulation of chitin synthetase in P. blakesleeanus (Herrera-Estrella and Ruiz-Herrera, 1983) mentioned above. In both wrapped and illuminated fermentors only vegetative growth occurred and there was no microscopic evidence of increased differentiation of the hyphae in the illuminated culture. This was most likely an effect of mixing because gravitational orientation is also important for sporangial development (Bergman et al., 1969). The phototrophic response was also noted for all Rhizopus species on solidified media, but was not always obvious when they formed short turfs on which the response was difficult to detect. Sporulation was also greatly improved when the organisms were grown in the presence of light, which led to the standard procedure of incubating cultures on solid media within 1 m of a 40 W incandescent lamp.

As a major metabolic product carbon dioxide affects growing fungi in numerous ways, including inhibition of growth, and the yeast-mycelial dimorphism of Mucor sp. noted above. The roles of carbon dioxide in fermentations were extensively reviewed by Jones and Greenfield (1982). Inhibition of mould growth occurs at quite low partial pressures (e.g. 0.002 atm results in a 10%

decrease of the growth rate of Aspergillus sp., while inhibition of P. chrysogenum occurs above 0.04 atm). The 20% inhibition of the growth rate of R. javanicus upon addition of 2% CO_2 to the air supply observed in the Versatec fermentors is therefore within the range of inhibitions observed for other moulds. Furthermore, in most fermentations slow nucleation of CO_2 bubbles limits the rate of desorption, so many broths are supersaturated. This will be discussed in connection with aeration, mixing and scale-up (Section 4.1.3).

Following the exponential period growth slowed and finally ceased; in preliminary media in Versatec fermentors a very sudden cessation was observed, but more gradual slowing was observed in high-salts media. If glucose remained, the slowing of growth was accompanied by the commencement of acid production (no bases which could be metabolized, such as ammonia, were included in the medium formulation). The acids produced were not identified, although R. javanicus is known to produce organic acids including fumaric acid (Inui et al., 1965). The fact that acidic equivalents were produced more efficiently (more moles of titratable acid equivalents were produced per mole of glucose consumed, Table 3.7) when the medium pH was high indicates that either a multifunctional acid, or more than one type of acid with only one carboxyl group, was being produced. The result of the acid production was that if the pH was not controlled it dropped much more quickly in cultures grown at higher pH values or at higher biomass concentrations than it did in cultures grown at lower pH values or in more dilute suspension. In either event the pH levelled off at about pH 2, at which point growth was very

slow (the pK_a values of common organic acids are greater than two, so continued acid production would not cause a further drop in pH). These dynamics must be kept in mind when interpreting all growth results. The observation of continued acid production at low pH by R. javanicus is different from the behaviour of Mucor rouxii which only produced acid above pH 7 when grown in a complex medium (Bartinicki-Garcia and Nickerson, 1962b).

Slowing and halting of growth is in general due to either depletion of an essential component for growth or accumulation of toxic or inhibiting compounds. The cessation of growth due to depletion of one or more trace minerals from the medium at very low biomass concentrations in preliminary media A and B is discussed in Section 4.2. Accumulation of a growth-inhibiting compound was not investigated, although the production of siderophores (Holzberg and Artis, 1983), which would have complexed essential metals (particularily iron) in the medium, might have resulted in growth patterns during the "feeding" of trace metals experiments similar to those which were observed. This is a dubious explanation for several reasons. Probably very little siderophore was produced in the 1-3 day old cultures in this work as significant production of these compounds by R. arrhizus and R. oryzea was reported to have occurred after extended periods of time in culture (eight weeks) (ibid), and production by R. oryzea was not observed at all in human serum (Rastinejad and Artis, 1984). As well, siderophore production is supposed to result in enhanced fungal growth rather than the contrary (Lavie and Stotzky, 1986). In any case, had siderophores been secreted into the solution, one would not have

expected the correlation between medium trace metals content and the biosorptive yield, which is a property of the solid, which was observed in this work.

Other components which are important for the organism's metabolism are oxygen and carbon dioxide, which must be transferred to and from the growth medium respectively. Gas transfer and agitation is discussed in Section 4.1.3 in connection with scale-up of biosorbent production.

To illustrate the importance of understanding the dynamics of the growth in culture when interpreting experiments, the shake flask study of the effects of inoculum levels and liquid volume on biosorbent production will be discussed. As illustrated in Figure 3.5 a 2-fold difference in the surface to volume ratio in 250-mL Erlenmyer flasks was important in determining both the final biomass concentration and the subsequent metal uptake behaviour (all of the flasks were harvested at the same time, after they had reached pH 2). The rates of oxygen absorption, carbon dioxide desorption and shear were all lower in flasks containing 100 mL than in those containing 50 mL. R. javanicus biomass propagated in 100 mL broth grew more slowly than the mycelium in the lower medium volume, but it achieved a higher biomass concentration, and underwent a more rapid decrease in pH when growth slowed and the metabolism switched to acid production. The biomass in the flasks containing 50 mL grew rapidly initially, but experienced much earlier onset of acid production, and at low biomass concentrations experienced slower

decreases in pH. In general, at pH 2 the mould did not grow, and changed its metal uptake behaviour extremely slowly, whereas at intermediate pH values biosorbent was produced which reflected the current rate of growth. The correlation between growth rate and metal uptake behaviour is discussed in Section 4.1.4, but it is sufficient to say here that the copper uptake ability was generally greater when the mycelium was propagated at a higher average growth rate during active growth. This experiment, which must be interpreted in light of knowledge of the dynamics of growth, highlights the fact that the behaviour of the organism must be thoroughly understood to avoid arriving at a totally erroneous conclusion (ie. that in general the copper uptake behaviour may be improved by growing the mould with a lower aeration rate). This false conclusion would be a consequence of the fact that all the flasks were harvested at the same time, after the period of active growth.

The inoculum level did not have as large an effect on biosorbent production in shake flasks as did the medium volume, although at 10^7 spores/L pellet formation was more pronounced and biomass production was lower. An inoculum level of at least 10^8 spores/L would be recommended for this organism growing under these conditions. This is a higher level than the 10^6 spores/L which Bartinicki-Garcia and Nickerson (1962b) found resulted in good growth of Mucor rouxii in a complex medium. However, they routinely used a higher inoculum level more in line with that which was found to be necessary here. In further studies of propagation of Rhizopus biosorbents one might wish to grow pellets because this form of growth results in better oxygen

transfer in a fermentor (Brierley and Steel, 1959). As well, pellets could be used directly as a recyclable ion exchange material (Treen-Sears et al., 1984). Pelletization was found to be affected by both the inoculum level and ethanol in the growth medium.

4.1.3 Scale-up of biosorbent production

When scaling up production of microbial biomass both gas transfer and agitation must be considered.

Oxygen demand by a growing mould is a function of the rate of growth and the efficiency of oxidation of the substrate. Rates of oxygen uptake for a variety of fungi ranging from 0.3 mmol/g dry weight/h for the yeast Debaryomyces formicarius growing at 0.11 h^{-1} (Panikov and Bondarenko, 1981) to 6 mmol/g/h for Candida utilis growing at 0.8 h^{-1} in non-limiting oxygen concentrations (Vorob'eva and Filomenko, 1982) have been reported. In the latter case an oxygen transfer rate of 0.6 mmol/L/min was necessary to avoid oxygen limitations. Intermediate oxygen uptake rates of 1.1 mmol/g/h to 2.1 mmol/g/h have been reported for the filamentous fungus Neurospora crassa and the yeast Saccharymyces cereviseae (Giese, 1973, p 438). Assuming the maximum rate, in a culture of 1 g/L the oxygen demand would be almost two-thirds the maximum oxygen transfer rate of 0.18 mmol/L/min measured by sulphite oxidation at an air flow rate of 2.2 vol/vol/min in the Versatec fermentors, and would be greater than the rate of transfer at lower air flow rates. The sulphite oxidation measurements cannot be related directly to oxygen transfer in the growth medium because the presence of disperse mould mycelium in the broth has been found

to decrease profoundly the oxygen transfer rate (up to 50% at 1.5 g biomass/L) as bubble dispersion and coalescence was affected by the solid (Brierely and Steel, 1959). Clearly oxygen limitation in the Versatec fermentors at low air flow rates and high growth rates could occur at very low biomass concentrations (less than 1 g/L). As well, organic components, particularily surfactants which manifested themselves in this work as foaming occurred late in the growth experiments, affect the rate of oxygen transfer (Bull and Kempe, 1971; Eckenfelder and Barnhart, 1961). However, there is no general rule regarding the effects of surfactants on oxygen transfer.

Oxygen transfer cannot be considered in isolation from agitation and carbon dioxide desorption from the medium. The growth rate of Mucor javanicus has been found to be directly related to the rate of agitation (Tanaka and Ueda, 1975). The shear rate experienced by Aspergillus niger (a septate mould) has also been reported to affect the growth rate, cell morphology and cell wall structure as shear decreased the sensitivity of the wall to lytic enzymes (Ujcova et al., 1980; Musilkova et al., 1981). As well, the rate of desorption of carbon dioxide from fermentation media is often limited by the rate of nucleation of bubbles and is therefore enhanced by increased agitation and air flow rate (Jones and Greenfield, 1982). In this work a 20% decline in the specific growth rate was observed in medium B when 2% CO_2 was added to the air stream (Table 3.7).

Halting of acid production and resumption of growth upon increasing the air flowrate in the "Versatec" fermentors was demonstrated with low initial aeration rates (0.5 L/min;

0.3 vol/vol/min), but at a rate of 4 L/min (2.7 vol/vol/min) constant growth in the high salts medium at a rate of 0.23 h^{-1}, from biomass concentrations of 0.03 g/L to 3 g/L was observed. These were both a high rate of growth, and the highest biomass concentration attained in this type of fermentor, and the fact that growth did not slow with increasing culture density indicates that aeration was adequate. Thus oxygen transfer was not a cause for concern in other experiments with this equipment at lower culture densities with the same air flow rate. However, it should be noted that the dissolved oxygen concentration in the broth was not measured.

The mould grew more quickly and a higher final biomass concentration was attained in the large, baffled, impeller-agitated New Brunswick fermentors used to propagate large quantities of biomass than in the Vibramixer-driven "Versatec" fermentors (5.2 g and 61 g acid-washed biomass/10 L broth after 26 and 44 h of growth compared to 0.06 and 3 g/L after 26 and 40 h). The increased growth rate was not due to improved aeration, because adequate aeration was provided in the Versatec fermentors. However, as discussed above, increased shear stresses within the liquid may have affected the growth rate.

4.1.4 Effect of growth rate on biosorbent quality

The effect of growth rate alone on biosorbent quality is difficult to assess, because there must be different growth conditions causing the differences in growth rate. Variation of the dilution rate in continuous culture is a common method of controlling growth rate while holding other parameters

essentially constant, but the Rhizopus moulds do not lend themselves very readily to this mode of cultivation. After 3 to 4 days in the fermentor they exhibit a great deal of wall growth, form a thick broth which does not behave properly in an overflow even at very low biomass concentrations, and grow through all orifices (including those on pH probes) and up tubes such as air inlet and sampling tubes, regardless of their size. They also attach themselves to every small discontinuity in the fermentor and start to form lumps which if left undisturbed may achieve impressive dimensions. Obviously one needs a different strategy.

The comparison of similar biosorbent samples which, because of relatively small differences in their growth conditions, grew at different rates, illustrates that there was a general trend of variation of metal uptake with growth rate. Three conditions which individually led to variation in growth rate were intensity of light, medium pH, and time of harvesting of biomass. When the copper uptake at 1.2 - 1.8 mM concentration in solution was compared, under two of these three conditions, the sample which was growing at a greater rate took up the most metal per gram of biomass. That is, the sample grown at optimum medium pH=5 (Table 3.9) and the younger samples of several Rhizopus sp. (Tables 3.6 and 3.11) took up more metal than the sample grown at pH 6 and the older samples respectively. However, there was no consistent trend among the samples obtained during the fermentations in the light and the dark (Table 3.8) because the copper uptake went through a maximum at different times. In general, among a variety of organisms, the pattern of metal uptake variation with culture age is not predictable. Biomass of the capsulated

bacteria Zoogloea ramigera exhibited an age-dependent maximum in copper and cadmium uptake (Norberg and Persson, 1984), while little difference in cadmium uptake with culture age of the bacteria Parracoccus sp. and Pseudomonas putida has been observed (Bollag and Duszota, 1984). However, higher uranium uptake by young mycelial cultures of Penicillium spp. than by older ones has been measured (Zajic and Chiu, 1972). As well, decreases in uranium uptake ability of R. arrhizus biosorbents with increasing time spent in culture have been made observed (Treen-Sears et al., 1984). Analysis of the results presented in that paper reveal that the biosorptive yield was constant during the period of growth which was examined.

Although it was originally thought that the copper concentrations of 1.2 - 1.8 mM used in these assays would result in near-saturation of the biomass, this was not the case when the strength of binding was low, as was repeatedly demonstrated in studies of metal uptake. Thus the entire metal uptake curve should be considered. In order to compare copper sequestering by a variety of samples the metal uptake was modelled in terms of an equilibrium (Treen-Sears et al., 1984), assuming one type of site. This was not rigorous with such a complicated material, but described the data very well and allowed direct, meaningful comparison of samples. When grown in dilute suspension, six Rhizopus species exhibited a decrease in "W" (the metal uptake observed at saturation of the biosorbent) and an increase in "k" (apparent strength of binding) with increasing time in culture (decreasing growth rate) (Table 3.11), as did the young and old HSM biosorbents (Table 3.14). Rhizopus javanicus biosorbents

which had been propagated in the light and the dark and harvested at the time of commencement of acid production exhibited a similar trend; the biosorbent propagated in the light, which had grown at a greater rate, exhibited a higher "W" and a lower "k" compared to that grown in the dark. The strength of binding was not measured for the samples propagated at different pH values.

Another situation in which the growth rate was changed was the experiment in which a trace-metals mixture was added to an old, stationary-phase culture. This caused a temporary (5 h) increase in the copper uptake by the biosorbent, which corresponded to the microscopic observation of initiation of fresh growth of the hyphae. This pointed out the possibility of alteration of the biosorbent behaviour by initiating a short period of growth in a stationary culture. If biomass which has been propagated for some other purpose (eg. synthesis of organic compounds) is subsequently to be used as a biosorbent, it may be worth carrying out a short treatment such as this in the fermentor, and timing harvesting to optimize the metal uptake characteristics. It might be considered surprising that such a large change in metal uptake behaviour of the biosorbent was observed as growth was initiated in the biomass, considering that little new material was actually incorporated into the mycelium. However, the formation of new growing tips involves extensive lysis and rearrangement of material within the existing cell walls (Gooday and Trinci, 1980). These changes in both the composition and structure of the existing material would contribute to the observed changes in copper uptake behaviour.

In summary, a generally observed trend was that, under similar conditions of growth, the strength of copper binding decreased and the total number of uptake sites increased with increasing rate of growth. When the media compositions were different, however, these trends were not followed, as was most vividly demonstrated by the effect of variation of the predominant divalent cation in the growth medium on subsequent metal uptake behaviour of the biosorbent (Figure 3.11). This is discussed further in Section 4.3.6.

4.2 Development of a Growth Medium for Copious Biomass Production

In section 4.1 the pattern of mycelial growth in suspension culture was described. The fact remained, however, that the biosorbent concentration in the growth medium was low (often less than 1 g/L), and this was the next problem to be addressed.

The strategy of the series of experiments leading to the development and study of the high-salts medium for copious production of R. javanicus biosorbents was related in the Results section and will not be repeated here. This was done for the sake of continuity, because the results of these experiments were self-evident and the conclusions from one experiment led to the design of the next. The results of this series of experiments are discussed below.

The observation that the trace metals content of the growth medium was directly related to the biosorptive yield of R. javanicus suggested that the growing mould was sequestering essential elements from the growth medium to such an extent that it was limiting its own growth. Similar limitations of growth

have been observed in activated sludges which contain microbes which either sequester metals themselves or secrete compounds which complex them, making them unavailable to the growing organisms (Callander and Barford, 1983a,b). In this case the slow production of biosorbent which continued after the commencement of acid production in the absence of pH control would be expected as the metals were displaced from the biomass by ion exhange reactions similar to those observed with a R. oligosporus biosorbent (Treen-Sears et al., 1984). Metal uptake by ion exchange has been observed for a broad range of algal, bacterial and fungal biomass samples, as was related in the Introduction, and is considered to be the chief mechanism of uptake by the biosorbents in this work (Section 4.3.4).

The fact that toxicity (slower growth) was observed when additional trace metals were present initially in the growth medium led to the batch feeding experiments, which supported the hypothesis that the mould was limiting its own growth by sequestering essential elements from the growth medium. Additional evidence of toxicity of the original stock trace metals mixture during the feeding experiments made obvious the necessity of the toxicity studies. Extreme toxicity of copper, nickel and cobalt is a common phenomenon among moulds (Somashekar et al., 1983), and was observed in this work as well. Fungi may adapt themselves to grow in normally lethal concentrations of most of the cations investigated (Bowen, 1979; Doelman and Haastra, 1984), but higher concentrations of less toxic cations may counteract or exacerbate the effects of more toxic metals (Mohan and Sastry, 1983). Therefore, the concentrations of the

metals which were found to reduce the quantity of growth in the toxicity experiment were kept very low in the high salts medium (HSM). The toxicity studies were carried out in media to which the organism was not adapted; in an attempt to mimic the trace metals feeding experiments the mould was adapted to the unsupplemented dilute medium C. The absence of growth in 2 M NaCl observed in this work may be a consequence of this lack of adaptation, as R. javanicus is reported to exhibit good growth in 5 M NaCl (Inui et al., 1965). It is possible that following adaptation the mould would grow well in higher concentrations of cations than appeared to be toxic in these studies. Nevertheless such a study points out the relative toxicity of a variety of ions.

The toxicity studies illustrated that the original trace metals mixture was poor, and adjustment of the composition led to a preliminary HSM which supported much better growth of the mould. Moreover, after it was determined that there was no antagonism of biosorbent production due to the levels of potassium phosphate, magnesium sulphate, and iron, zinc and manganese levels a HSM was devised which supported rapid growth in the "Versatec" fermentors (0.23 h^{-1} specific growth rate) leading to much higher biomass concentrations (3 g/L at the time of harvesting from the "Versatec" fermentors, and 8 g/L in the "New Brunswick" fermentor). The latter biomass concentration compares favourably with those achieved when growing R. arrhizus and R. nigricans to produce fumaric acid, for which concentrations of 6 g/L to 8.5 g/L were reported (Rhodes et al, 1959; Burnett, 1976 p 246). In these studies the concentrations of zinc, phosphate

and magnesium affected significantly the quantities of both biomass and acid in the final culture. The "biomass concentration" measured in this work was lower than a conventional dry weight assay (Section 3.2), so the final concentration of R. javanicus propagated in the HSM was actually higher than those reported in the literature.

4.3 Effects of Growth Conditions on Biosorbent Quality

Studies of the effects of age in culture and the predominant divalent cation in the growth medium on subsequent metal uptake behaviour demonstrated that metal uptake behaviour by biosorbents derived from a single species may be profoundly affected by the conditions under which it was propagated. However, they shed little light on the reason for these variations. In the final set of experiments large quantities of biosorbent were propagated under three different conditions. They were grown for short and long times in the high salts medium to produce young and old HSM biosorbents, and to a low biomass concentration in a medium with a lower level of magnesium, and higher concentrations of calcium and zinc, HCZ. These samples were acid-washed with 0.01 M HCl to produce standard biosorbents comparable to those studied previously, and were also extracted with lithium chloride and nitrous acid at room temperature. The chemical composition of the standard and extracted biosorbents, as well as their uptake of copper (II), manganese (II) and chromium (III) from solutions of single salts over several orders of magnitude of solution concentration, were determined. Slurries of the old HSM biosorbents were also titrated with sodium hydroxide.

The objective of these experiments was to provide information which would allow elucidation of the relationships between the conditions under which the biosorbent had been propagated and the subsequent metal uptake behaviour. The discussion of these relationships will be divided into a series of steps. The biosorbent chemical composition will be examined, and information from other studies of the synthesis of cell wall components will be reviewed. Following this the structure of the biosorbent will be discussed, and only then will the metal uptake behaviour be examined and related to the information on the biosorbent make-up.

4.3.1 Biosorbent chemical composition

The biosorbents contained, by weight, 8-12% phosphate (PO_4), 23-26% uronic acid (glucuronolactone basis), 28-37% glucosamine and 14-43% protein, and despite differences among the samples, their chemical compositions were typical of mucoralean cell walls (Bartinicki-Garcia and Nickerson, 1962c; Bartinicki-Garcia and Reyes, 1968a,b; Datema et al., 1977a,b; Dow et al., 1983). The correspondence between the biosorbent assays and cell wall assays, along with the microscopic observation of freedom from cytoplasmic contamination and the substantial weight losses during extraction (Section 3.4.1), indicate that the biosorbents were principally cell wall material (Bartinicki-Garcia and Nickerson, 1962c). The procedure used to purify the biosorbent would result in a cell wall preparation which was not necessarily free of lipids, although their absence is not considered essential for "pure" mould cell wall preparations (Burnett, 1976 pp 19-21). Lipids were not expected to participate in metal uptake, so in this work the lipid content of the biosorbent was not assayed,

although it is reported to represent 5%-8% of the cell wall material of Mucor rouxii (Bartinicki-Garcia and Nickerson, 1962c; Bartinicki-Garcia and Reyes, 1968a). The high phosphate levels often observed in isolated mucoralean walls, and observed here as well, were once thought to be due to cytoplasmic contamination during purification. However, X-ray microprobe analyses and evidence that the phosphate is firmly bound and is impervious to prolonged washing has convinced investigators that it is a true cell wall component (Campos-Takaki et al., 1983). Many of the reports in the literature of mucoralean cell wall composition, structure and synthesis are examinations of the walls of Mucor rouxii and M. mucedo, and originally stem from investigations of the yeast-mycelial dimorphism of these organisms. However, Zygomycetes are all thought to exhibit the same general mechanisms of cell wall synthesis because the wall composition is similar among genera (Bartinicki-Garcia, 1968), as is the structure (Sengbusch et al., 1983).

4.3.2 Synthesis of cell wall components

When examining the influence of the growth medium composition on the cell wall make-up it is convenient to start by examining the synthesis of the cell wall polysaccharides, particularily chitin (poly-N-acetyl glucosamine) and chitosan (unacetylated chitin). In practice these polymers are rarely pure and the terms usually describe substances which are predominately acetylated or deacetylated respectively. In the following arguments they are used to refer to the appropriate pure fractions of the glucosamine polymers. Chitan is the name for

fully acetylated chitin, but this term will not be used, in recognition of the fact that this material does not occur in these fungal walls. Before examining the observations in this work, synthesis of individual cell wall polymers will be reviewed.

Chitin synthesis has been studied in vitro using particles called chitosomes, as well as subunits thereof, which have been isolated from several species of fungi (reviewed by Gooday and Trinci, 1980). Chitin synthetase has an absolute requirement for a divalent cation, and Mg^{2+} is most probably the one acting in vivo. In vitro stimulation of virtually all enzyme preparations occurred in the presence of magnesium at 10 mM concentration, and for some manganese was as effective at a low concentration (5mM), but inhibitory at higher concentrations. In various preparations cobalt was also stimulatory, or at least not inhibitory, to chitin synthetase, and iron (III) was also slightly stimulatory. With magnesium in the reaction mixture substantial inhibition of the enzyme was observed when zinc was added; barium was less inhibitory and calcium was even less so (ibid; Lopez-Romero and Ruiz-Herrera, 1976; Peberdy and Moore, 1975; McMurrough and Bartinicki-Garcia, 1971; Huizar and Aronson, 1985).

Chitosan synthesis in Mucorales is a two-step process in which chitin is formed and subsequently deacetylated to a greater or lesser degree to form chitosan. The deacetylase has no requirement for a divalent cation, but is inhibited by acetate (an end product) and acts only on polysaccharides (Araki and Ito, 1974). A chitin synthetase plus chitin deacetylase enzyme mixture for in vitro preparation of chitosan exhibited the same requirement for a divalent cation exhibited by the chitin

synthetase alone. Magnesium was stimulatory over a broad range of concentrations, and manganese was stimulatory at low concentrations and inhibitory at high concentrations. Calcium, zinc, iron (III) and copper (II) each inhibited the enzyme mixture; calcium had the smallest effect, copper had the greatest and zinc and iron had intermediate effects in the order listed. Sodium, potassium and ammonium had little effect on chitosan formation (Davis and Bartinicki-Garcia, 1984a). Although the factors which control the extent of deacetylation of the chitin are not well understood, it is thought that the crystallinity of the nascent polymer may play a role. In a reaction mixture *in vitro*, intact chitosomes exhibited poor formation of chitosan, but when the enzyme complex was dissociated so that nascent chitin could not immediately crystallize into microfibrils, the acetyl residues were accessible to the deacetylase and marked improvement in chitosan formation was observed (Davis and Bartinicki-Garcia, 1984b). This work led to the hypothesis that deacetylation of chitin within the cell wall was controlled by the degree of organization of the chitin synthetase on the cell surface.

The formation of uronic acid polymers in mucoralean cell walls has been investigated much less than chitin and chitosan synthesis. However, *in vitro* studies using crude membrane fractions from *Mucor rouxii* which possessed glucuronosyl transferase activity have demonstrated an absolute requirement by the enzyme for a divalent cation; 4 mM manganese was the most effective and magnesium was one-third as effective (Flores-Carreon *et al.*, 1985). Inhibition was not reported.

In this work the inhibition of chitin synthesis in the HCZ medium was clearly demonstrated; the glucosamine content of the standard biosorbent was lower. As well, phosphate levels were lower and uronic acid levels were higher in the HCZ biosorbents than in the HSM biosorbents. The balance of anionic and cationic residues within the cell wall is important to its structure and must be maintained. If the majority of the ionizable species are polysaccharides and phosphate, then when the rates of production of chitosan and uronic acid polymers are fixed by the divalent cation content of the growth medium, the only remaining variable is the quantity of ionizable phosphate to be incorporated in the structure. This would explain the observed shift in the quantities of anionic residues within the HCZ biosorbents compared to the HSM samples. Presumably the glucuronosyl transferase was not affected in the same manner as the chitin synthetase. It is possible, however, that variations in the quantity and quality of protein may also play a role in this instance. Growth in the HCZ was slower and the hyphae were on average about 50% larger in diameter than those in the HSM. Similar variations in the hyphal morphology were observed in the shake flask studies of the effects of magnesium and calcium on biosorbent production. As well, the recovery of cell wall material from the water-washed biomass was lower than in the case of the HSM biomass, all of which indicates that there was a shortage of cell wall material compared to the volume of cytoplasm. Protein levels were between 2.5 and 3 times higher in the standard HCZ biosorbent sample than in others, which suggests that it was used to reinforce the cell walls when chitin and

chitosan were in short supply. This finding corresponds to that of Mauro and Dietrich (1981) who found that pro ein levels in cell walls of Mucor javanicus were increased 80% when the mould was propagated in a complex medium containing calcium rather than the synthetic Mucor medium which supposedly contained no calcium (the inoculum was propagated on potato-dextrose agar, so some calcium would have been carried over during inoculation).

The chitosan content varied among the samples, but those with both the highest and lowest degree of deacetylation of the glucosamine polymer were grown in the HSM. The growth rate or rate of chitin formation appeared to be the controlling factor; the near-stationary old HSM underwent the greatest deacetylation, while the very rapidly growing young HSM underwent the least. The biosorbent propagated in the HCZ medium, in which chitin synthesis was partially suppressed, exhibited an intermediate degree of deacetylation. As well. the degree of deacetylation of the glucosamine polymer varied inversely with the concentration of chitin in the acid-washed biosorbent. Whether the crystallinity of the nascent chitin in these samples was indeed different, as was previously postulated (Davis and Bartinicki-Garcia, 1984b), or the deacetylase enzyme system which only operates on the nascent polymer was the rate-limiting step in chitosan formation remains unknown. It is also possible that crystallinity of the hyphal tip was affected by cations in the medium as calcium causes increased rigidity of the tip while hydrogen ions (mineral acids) cause tip bursting (Gooday and Trinci, 1980). However, there was no indication in this work that calcium decreased the rate of chitin deacetylation, although

the effects of the acid on the filter cake and the susceptibility of the mycelium to homogenization in the Waring blender during biosorbent preparation were noted. Softening of Rhizopus biosorbents in contact with mineral acids has been observed previously during uranyl ion elution from a packed column of R. arrhizus beads (Treen-Sears et al., 1984).

4.3.3 Biosorbent structure

The structure of Mucorales cell walls containing positively-charged chitosan and negatively-charged phosphate and glucuronic acid residues is maintained at least in part by electrostatic interactions between ionizable groups. Both extraction solutions caused disruption of ionic binding within the materials; the lithium ion acted solely by interfering in the actual ionic bonds (Dow et al., 1983), while the nitrous acid reacted with the primary amine groups of chitosan, causing depolymerization as well as elimination of its charge (Datema et al., 1977).

One problem which arose in the course of attempts to relate the biosorbent composition to metal uptake behaviour was that the chemical analyses gave no information regarding differences in the structure of the materials. For instance, phosphate may be present in many different forms, only some of which participate in biosorption, and the total phosphate assay did not distinguish among these forms. Discrepancies between the quantities of potentially ionizable species present according to chemical assays and those available for ion exchange were evident during titrations of the old HSM biosorbent samples. The number of titratable sites was half the sum of the phosphate, uronic acid

and chitosan content according to the chemical assays. However, the maximum uptake of metals by the old HSM biosorbents, as calculated and tabulated in Table 3.14, was comparable to the cation exchange measured during titration to pH 6. The titrations of solids is a simple procedure and is common in investigations of materials containing ionizable groups. The cation-exchange capacity may be determined by displacement of alkali or alkaline-earth cations, or by titration, and is routinely quoted for commercial ion exchange resins. Determinations by titration have been carried out in studies of algal cell walls (Crist et al., 1981), bacterial cell walls (Beveridge, 1978), humic acids (Perdue and Lytle, 1983), peptides (Saudek, 1981) and chitosan samples (Masri and Randall, 1978; Muzzarelli et al., 1981a; Park et al., 1984).

4.3.4 Mechanism of metal uptake

As was described in the Introduction, metal uptake by materials of biological origin has been ascribed to many different mechanisms including active transport, ion exchange or complexation, adsorption of simple species as well as hydrolysis products, entrapment of colloids, and precipitation of insoluble compounds. Active transport may be immediately ruled out for the Rhizopus biosorbents examined in this work because it only occurs in living organisms. As well, the biosorbent preparation methods removed materials which might form colloidal metal products in solution. The other possibilities well be examined in turn, in light of others' work with similar systems.

It was with the intention of studying ion exchange phenomena that the ions copper (II), manganese (II) and chromium (III) were chosen for investigation. They undergo virtually no hydrolysis in solution at the pH of the metal uptake assays (Baes, 1976), so uptake would be due to ion exchange rather than adsorption of polynucleate hydrolysis products. The latter is probably what occurred during thorium deposition on the exterior of R. arrhizus cell walls (Tsezos and Volesky, 1982b) because thorium undergoes extensive hydrolysis to form polynucleate species and is extremely insoluble in the presence of phosphate (Cotton and Wilkinson, 1972). It is probable that chitin was not the major component on the exterior of the stationary-phase R. arrhizus cell walls studied by Tsezos and Volesky, and that phosphate, a major cell wall component, was present in significant quantities. During maturation of fungi the primary wall of the growing tip, which consists mainly of microfibrillar material (chitin), is covered by other amorphous components (Burnett, 1976, pp 81-84; Gooday and Trinci, 1980). As well, chitin was not exposed on the exterior of sporangiophores of R. nigricans (Sengbusch et al., 1983). Deposits of lead on walls of mucoralean fungi, which appeared to be similar to those of thorium, were ascribed to phosphate (Campos-Takaki et al., 1983).

The possibility of precipitation of insoluble compounds in the cell wall must also be considered. In studies of metal uptake by Bacillus subtilis cell walls a two-step process was proposed, in which cations were complexed by amine, phosphate or carboxyl groups and additional metal was deposited as microcrystals or aggregates to such an extent that more metal was

bound than could be accounted for by the numbers of polar sites within the biomass (Beveridge and Murray, 1980). The deposits were initiated by complexation by the carboxyl groups of peptidoglycan, and were observed for iron (III), cerium (IV), palladium (II) and gold (III). However, this is no basis on which to conclude that similar behaviour may have occurred in the present study. The materials which precipitated have a more complicated solution chemistry than the ions investigated in this study. As well, the quantities of metals taken up by the R. javanicus biosorbents were less than could be ascribed to complexation by ionizable groups, as assayed by chemical or titrimetric methods (Section 4.3.3).

Arguments were advanced by Tsezos and Volesky (1982a) that chitin was the cell wall component chiefly responsible for uptake of the uranyl ion by a Rhizopus arrhizus biosorbent. Uranyl ion uptake at pH 3-4 by chitin was 4.5-6 mg U/g, compared to 180 mg U/g by the biosorbent (ibid; Andreyev et al., 1962). With no other possibility available, the balance of the uptake by the R. arrhizus biosorbent was ascribed to adsorption and inorganic precipitation of uranyl hydroxide. Inorganic precipitation was said to occur at pH 4, and to result in slower accumulation of the uranyl ion, taking place after half an hour, on the basis of kinetic data. At pH 2 such precipitation was not supposed to have taken place, but equilibrium was not reached rapidly, as would be expected if the slow precipitation process was not occurring. This is not consistent. One of the main messages of this current work is that more than one type of site is responsible for metal uptake, either by complexation or ion

exchange, and under such conditions complex kinetics of uptake processes are possible (Helfferich, 1965). Tsezos and Volesky did not recognize that mucoralean cell walls contain large quantities of phosphate and glucuronic acid, and therefore ignored the possibility of extensive participation of these oxygen-containing ligands in metal binding. Their electron microscopic evidence of layering of the uranium deposits within the cell wall does not prove that chitin was extensively involved because the electron-dense layers could easily have corresponded to amorphous polyuronide, polyphosphate and protein which are interspersed between the layers of fibrillar structural polysaccharides (Burnett, 1976; Gooday and Trinci, 1980). Layers of lead deposited within mucoralean cell walls have been ascribed to binding with phosphate (Campos-Takaki et al., 1983).

In other studies of uranyl ion uptake by a variety of Rhizopus biosorbents (Treen-Sears et al., 1984) metal uptake was found to be due to ion exchange. In a packed column of acid-washed R. arrhizus biomass exchange of 1 UO_2^{2+}:2 H_3O^+ was observed, and a multiple-site equilibrium model described the patterns of metal uptake at different pH values and in the presence of competing ligands. Uptake at high pH in the absence of these ligands was reversible upon adjustment of the solution composition. Tobin et al. (1984) also concluded that uptake of a variety of metals by a R. arrhizus biosorbent was due to coordination with anionic residues. Thus it is evident that metal uptake by the R. javanicus biosorbents can reasonably be ascribed to ion exchange.

Table 4.1

Metal Uptake by Biomass Samples

Material	Metal Uptake Cu2+ (µmol M/g sorbent, unless otherwise stated)	Mn2+	Cr3+
R. arrhizus[1]	250	220	590
Mucor rouxii (untreated)[2,*]	56%	54%	59%
Mucor rouxii (boiled in 40% NaOH)[2,*]	100%	48%	84%
Aspergillus niger chitosan-glucan[3,*]	87-98%	82-40%	90-97%
chitin[4,**]	25.0%	-	47.8%
chitosan[4,**]	70.5%	-	53.4%
activated sludge[5]	150	-	690
chitosan[5]	3120	1440	460
chitosan, gluteraldehyde crosslinked[6]	570	110	660
chitosan[7]	2290	191	2.6
chitin[8]	60	-	-
chitosan[8]	180	-	-
carboxymethyl derivative of chitosan[8]	400	-	-
salicylidene-chitosan (substituted phenol)[8]	620	-	-
reduced sal.-chit. (less dense material)[8]	3030	-	-
Bacillus subtilis cell walls[9] untreated	2990	801	-
amine groups > electronegative	860	880	-
carboxyl groups > neutral	993	732	-
carboxyl groups > slightly electropositive	506	680	-
carboxyl groups > very electropositive	260	100	-
minus teichoic acid (PO4)	2488	650	-

Table 4.1 continued

Rhizopus javanicus biosorbents[10]			
young HSM, acid-washed	330	120	115
young HSM, LiCl-extracted	225	104	75
young HSM, HNO_2-extracted	180	80	51
old HSM, acid-washed	290	200	140
old HSM, LiCl-extracted	170	140	103
old HSM, HNO_2-extracted	130	50	50
HCZ, acid-washed	270	140	110
HCZ, LiCl-extracted	390	1300	250
HCZ, HNO_2-extracted	130	50	28

* uptake conditions: pH = 7.5; 200 mg solid/50 mL of 0.05 mM solution
values are percent removal of the metal ion from solution

**uptake conditions: pH = 6.5; 5 g sorbent/500 mL solution containing 1 mg of each of Cu, Zn, Cd, Cr and Pb per mL
values are percent removal of the metal ion from solution

1. Tobin *et al.*, 1984
2. Muzzarelli *et al.*, 1981b
3. Muzzarelli *et al.*, 1980a
4. Yang and Zall, 1984
5. Masri *et al.*, 1974
6. Masri and Randall, 1978
7. Yaku and Koshijima, 1978
8. Hall and Yalpani, 1980
9. Beveridge and Murray, 1980
10. sums of W's from Table 3.14

4.3.5 Roles of biosorbent components in metal uptake

Values of copper (II), manganese (II) and chromium (III) uptake by a variety of biomass samples and polymers described in the literature are compiled in Table 4.1. The saturation uptake values calculated for the biosorbent samples studied in the final section of this work (sum of W's, Table 3.14) are also presented. This is useful, both for comparison of the performance of various biosorbents, and deduction of the roles of groups within the biosorbents during metal uptake. In many instances the values reported in the literature did not represent saturation of the material; rather uptake under similar conditions was reported for comparative purposes. Thus the percentage removal of the metal ion from solution under set uptake conditions was reported for some samples. As well, the metal uptake reported by other authors was generally measured at pH values of 6-7.5, which are much higher than pH~3.5 in this study. It is evident that metal uptake values comparable to or much greater than those observed in the course of these studies have been observed for other biological materials and their derivatives, particularily for the Bacillus subtilis cell wall and chitosan. The former was discussed above. Uptake by chitosan is considerably diminished below pH 5; at pH 3.5 it is decreased by a factor of four, and at pH < 3 it is almost negligible (Yaku and Koshijima, 1978).

The information on metal uptake by Mucor rouxii was included because it is supposed to represent the enhancement of metal uptake ability (uncorrected for weight loss in preparation) after boiling the mycelium in sodium hydroxide to remove other polymers and deacetylate the chitin. However, the untreated mycelium was

only rinsed with water, dried and ground. Undoubtably large quantities of soluble components from the cytoplasm were therefore present in the metal solution, and these compounds could have interfered with metal uptake. These materials would have been removed in the course of the hydroxide treatment, which might account for the apparently enhanced metal sequestering abilities of the boiled mycelium. In any case, the enhancement of copper uptake relative to manganese uptake following the hydroxide treatment demonstrates that metal uptake behaviour typical of chitosan was bestowed in this way. Copper has a much greater affinity for these nitrogen-containing groups compared to manganese or chromium, which are bound extremely weakly (Park et al., 1984). This was clearly demonstrated by both by the metal uptake data for chitosan, and the shift in uptake by Bacillus subtilis cell walls upon modification of the amine groups.

In complicated materials such as the Rhizopus biosorbents, several types of ionizable sites affect the metal uptake capacity: phosphate groups; carboxyl groups on uronic acids and proteins; and nitrogen-containing ligands on proteins as well as, of course, on chitin or chitosan. Ion exchange behaviour would change as the predominance of various species or structure of the network of polymers was affected by the conditions of growth.

The three metallic cations investigated did not in all instances interact with the biosorbent in the same manner. Had all of the metals been bound to the same type of site, the maximum chromium uptake would have been two-thirds that for both manganese and copper (Table 4.1). This was the relationship observed for the lithium chloride-extracted old HSM and HCZ

biosorbents when the number of sites for copper and chromium uptake were compared. On the other hand, typical ion exchange behaviour was observed for six of the nine biosorbents when the manganese and chromium values of the sum of W's were compared. Anomolously high values of the sums of W's for copper were most probably due to its strong affinity for nitrogen-containing groups, discussed above.

It is difficult to predict metal uptake behaviour with complicated materials such as cell walls because the configuration of binding sites within the cell wall may confer a degree of specificity. This was discussed by Gale and Wixson (1979) in connection with sequestering by algal cell walls for which the order of selectivity did not correspond to the charge density of the cations. As well. the stability of complexes may be a function of the ligand mixture when a variety are present in the biosorbent matrix (ibid). The preferential binding of copper by amine groups is one example of this. Furthermore, in a mixed polymer of acetylated and unacetylated glucosamine, copper may be bound to either one or two amine groups (Park et al., 1984). Copper taken up by chitosan membranes has also been found to associate with one or two amine groups, depending upon the pH (Muzzarelli et al., 1980c). Flexibility of metal binding by bacterial cell walls has also been observed as anionic groups paired with one or two cations, depending upon the solution composition (Marquis et al., 1976). Another example of the significance of particular ligands in a complicated material was demonstrated by the binding of uranium at pH 2.5 by Penicillium biomass, which was ascribed to the presence of strongly acidic

groups such as phosphate (Galun et al., 1983a). Tobin (1986) ascribed uptake of a variety of cations to ion exchange by oxygen-containing ligands in a Rhizopus arrhizus biosorbent, and described binding by hypothetical sites of configurations which were suited to ions of different sizes.

Although it would be desirable to assess quantitatively the contribution of each of the different groups or types of sites to metal uptake, only one or two theoretical sites were needed in each case to model mathematically the metal uptake behaviour (Table 3.14). This indicates that there were not large enough differences in the equilibrium constants, or that one or two groups predominated. There was also heavy coupling between the calculated equilibrium constants and concentrations of sites on the biomass, so the values were of descriptive use but not of physical significance. Similar problems have been observed previously by Perdue and Lytle (1983) who tried to model metal uptake by humic acids in the same manner. Nevertheless, it was possible to make several qualitative observations which clarified the roles of various biosorbent components in metal sequestering.

Consideration of the nitrous acid-extracted biosorbents led to two conclusions regarding the roles of chitin and chitosan in metal uptake. These samples had higher levels of chitin and lower levels of uronic acids and phosphate than the corresponding acid-washed or lithium chloride-extracted biosorbents, and also exhibited a much lower metal uptake capacity. Thus the phosphate and uronic acid residues were more important than the glucosamine components for the sequestering of these metals. This conclusion

was also supported by the marked similarity in the metal binding by the standard young and old HSM biosorbents, despite the large differences in both the quantity and the quality (degree of acetylation) of the glucosamine polymer.

The second conclusion regarding the roles of chitin and chitosan in metal uptake arises from the fact that the chitin in the nitrous acid-extracted biosorbents was totally acetylated. All cationic amine groups within the biosorbent matrix would be removed by this extraction treatment (Datema et al., 1977), and it was evident upon examination of the titration curve of the nitrous acid-extracted old HSM biosorbent that these groups, which are titrated at a higher pH than carboxyl or phosphate residues (Skoog and West, 1976, pp 784-5) had been eliminated. When both anionic and cationic groups were present in the biosorbent matrix the apparent binding constants (Table 3.14) were low (40-80 mM^{-1} for the stronger types of sites on the acid-washed biosorbents). This could correspond to the copper binding constant of 110 mM^{-1} for chitosan, but binding constants for other metals are considerably lower (Park et al., 1984). For the nitrous acid-extracted samples, which did not contain fixed positively-charged amine groups on the chitosan, the binding constants for the strong types of sites increased by an order of magnitude (300-1000 mM^{-1}). Although these values were still low, they then approached the values of constants for complexation by phosphate or carboxyl groups in free solution (log (k) for polyphosphate complexation of Mn^{2+}, Cu^{2+} = 5.5; log (k) for citric acid binding of Mn^{2+} = 3.7 and of Cu^{2+} = 5.9; log (k) for acetic acid binding of Cu^{2+} = 2.1) (Callander and Barford, 1983b;

Skoog and West, 1976, p 786). This points to the strong possibility that weaker binding by the acid-washed and lithium chloride-extracted samples compared to the nitrous acid-extracted ones was because of simultaneous interaction of the cationic residues in the biosorbents with both the anionic residues in the biosorbent and the metal ions. Rigidification of the cell wall has been in part ascribed to electrostatic interaction between these residues, so they would be in close enough proximity for simultaneous interaction with the metal ions. This would account for the observations of Tsezos and Volesky (1982a,b) that amine groups were involved in metal binding, even if they were not the primary reason for its occurrence.

The roles of phosphate and uronic acid residues were elucidated further by studying variations in biosorbent composition and metal uptake behaviour among acid-washed and lithium chloride-extracted samples of the same biomass. There was no general correlation between the ratio of uronic acid to phosphate and the relative strengths of binding of the metals. None was found because of the limitations of the chemical assays with regard to structural information. However, a common trend was observed upon comparison of standard and lithium chloride-extracted samples of any single type of biosorbent. Consider first the young HSM biomass, which was the most refractory; its composition was changed the least by the extractions. When the acid-washed and extracted biosorbents are compared, it is evident that lithium chloride extracted primarily phosphate, along with some uronic acid. This caused a shift in the relative uptake of the three metals at concentrations between 0.01 and 1 mM. Copper

was sequestered by the extracted sample in a manner similar to the acid-washed biomass, but manganese uptake was decreased to levels slightly greater than or comparable to the chromium uptake. Similar, but much more dramatic shifts in relative uptake of different metals were observed for extracted samples of the old HSM and HCZ biosorbents, to the extent that more chromium than manganese was bound by the extracted HCZ sample. This sample exhibited very weak binding of all metals. Thus the absolute strength of binding of individual metals, as well as the relative strengths of binding of different ones, were affected as the ratios of uronic acid to phosphate were increased by lithium chloride extractions.

The role of protein in metal uptake by biosorbents was not studied extensively, as no isolation or characterization of proteins was carried out. In fact the composition of the proteins of the cell wall may be altered by growth conditions, as was found for Mucor rouxii (Dow and Rubery, 1977). The standard HCZ biosorbent contained much higher protein levels than the other samples, and if protein was responsible for a large fraction of the metal binding this sample should have exhibited a correspondingly greater metal uptake, which it did not. The pattern of metal uptake by the acid-washed HCZ biosorbent was different from the other two acid-washed samples, and the extent to which this may be attributed to the protein itself or to differences in the molecular arrangements or structure of the material as a whole (which would be affected by the protein) was not determined.

4.3.6 Relationships between growth conditions and subsequent metal uptake behaviour

During the course of these studies many unextracted biosorbent samples were examined, and elucidation of the relationships between the growth conditions and the subsequent metal uptake behaviour is the culmination of this work.

To summarize the above arguments, the overall strength of binding of metal was affected by the cationic amine residues within the biosorbent; they interacted with both the metallic cations under investigation and the anionic ligands responsible for immobilization of the metals. The effect on the strength of binding was related to the degree of deacetylation of the glucosamine polymer and the configuration of the polymers in the biosorbent matrix. When chitin synthesis was inhibited in the presence of high concentrations of appropriate divalent cations, protein played a role in the structure of the cell walls, and with fewer fixed positive charges within the biosorbent metal binding tended to be stronger. The degree of deacetylation of the chitin increased as the rate of synthesis decreased, suggesting that this step was the rate-limiting one in chitosan synthesis *in vivo*. The ratio of phosphate to carboxyl residues could be influenced by the cation composition of the growth medium, or by extraction of the biosorbent with a lithium chloride solution. The relative strengths of binding of different metals were found to be related to this ratio, and may be related to the selectivity of the biosorbent for the metals. Such was thought to be the case for metal uptake by algal materials and a *Rhizopus arrhizus* biosorbent (Ferguson and Bubela, 1974; Tobin 1986).

The structure of the hyphal wall is known to change as the mycelium ages. Although the processes of rigidification are poorly understood the older or more mature wall is characterized by increasing stiffness and degree of crystallinity which is reflected in a decreased susceptibility to lytic enzymes (Gooday and Trinci, 1980; Gabriel, 1984). The increasing crystallinity corresponds to closer bonding between the anionic and cationic cell wall components, which in the biosorbent results in an increase in the apparent strength of metal binding and a decrease in the total number of uptake sites. This would occur because close association of the free amine groups with anionic residues precludes simultaneous interaction with metallic cations, and the remaining ionizable residues (in all samples analysed there was an excess of potentially anionic groups) within the biosorbent were free to bind metals in an unhindered fashion.

In future work it would be desirable to propagate biosorbents with given properties. This would involve exploitation of the differences in responses of chitin and poly-glucuronic acid synthetases to divalent cations, as they act as cofactors or inhibitors of the enzymes. As well, growth rate is a variable that affected the degree of chitosan formation and could be controlled independently, so there are enough variables to "tailor" a biosorbent to a particular situation. With the knowledge that the growth conditions affect so profoundly the behaviour of biosorbents, their potential awaits application.

5. Conclusions

Totally defined glucose/urea/mineral salts media for propagation of Rhizopus sp. have been described. One of these, a high salts medium, supported rapid growth of R. javanicus to a high final biomass concentration (>8 g/L) after 45 h.

Rhizopus sp. did not grow with nitrate as the sole nitrogen source on synthetic media. This confirms the observation of Inui et al. (1965) using other media.

R. javanicus did not grow under anaerobic conditions, and grew as a mould rather than as a yeast in sealed flasks under oxygen-limited conditions, in media containing concentrations up to: glucose, 130 g/L; ethanol, 20 g/L; $CaCO_3$, 100 g/L.

Variation of the morphology of R. japonicus grown on organic and synthetic media has been described. This is of taxonomic significance and illustrated the importance of repeated transfers to prepare mould inocula of stable reproducible morphology.

In synthetic media R. javanicus exhibited a maximum specific growth rate at pH 5.

Light affected cultures of Rhizopus sp. on solid media in that it was necessary for differentiation of sporangia and the sporangiophores exhibited positive phototropism. In suspension culture R. javanicus experienced a 50% increase in the specific growth rate during exponential growth in illuminated cultures compared with those grown in the dark. Under microscopic examination this was not accompanied by evidence of differentiation of morphology.

The toxicity of metals in a dilute synthetic medium decreased in the order: copper (II); cobalt and nickel; manganese (II) and molybdenum (molybdate); and chromium (III).

The chemical composition of R. javanicus biosorbents was affected by the divalent cation (esp. Mn, Zn, Ca and Mg) content of the growth medium. This was related to the roles of these cations as cofactors or inhibitors of enzymes for synthesis of cell wall components (chitin/chitosan and poly-glucuronic acid) which contain ionizable groups.

Uptake of Cu(II), Mn(II), and Cr(III) at pH 3.2-3.8 was affected by the biosorbent composition as it was varied by alteration of the growth conditions. Uptake was chiefly due to ion exchange or complexation with phosphate and carboxyl (glucuronic acid) residues within the cell wall, although chitosan amine groups participated in copper uptake.

When the biomass was grown at different rates in similar media by modification of pH, illumination or timing of harvesting, copper uptake by the biosorbent was affected. The strength of binding decreased and the number of sites increased as the growth rate increased. Variations in uptake behaviour were related to changes in the cell wall structure during maturation.

Metal uptake was affected by extraction of the biosorbent with lithium chloride. When compared to acid-washed biosorbents (unextracted), this resulted in an increase in the ratio of uronic acid to phosphate in the biosorbent, and an increase in binding of chromium compared to manganese.

The strength of binding of all metals investigated was increased by an order of magnitude after removal of amine groups by extraction with nitrous acid. This suggests that electrostatic interactions between chitosan, and acidic residues in the biosorbent and metallic cations, tend to decrease strength of binding by the biosorbents. The removal of the primary amine residues was evident upon titration of biosorbent slurries with sodium hydroxide.

6. Original Contributions

It is felt that original contributions have been made in two areas: the studies of the phenomena of biosorption, which may lead to exploitation of such substances; and the propagation and cell wall formation of Rhizopus sp.

6.1 Phenomena of Biosorption by Rhizopus

Relationships between the biosorbent composition and the metal uptake behaviour were elucidated:

- The relative strengths of binding of metal cations were affected by the ratio of phosphate to uronic acid residues.
- The strength of binding of metal cations was decreased when cationic amine groups on the chitosan were available to interact with the metallic ions.

Aspects of the effects on the biosorbent metal uptake behaviour of the growth medium composition (particularily the divalent metal content), the physical conditions of growth (in part as they affected the growth rate of the biomass), timing of the harvesting of the biomass, and/or extraction procedures used on the harvested biomass were elucidated.

6.2 Propagation and Physiology of *Rhizopus*

Totally defined glucose/urea/mineral salts growth media, one of which supported growth of *R. javanicus* to high biomass concentrations in suspension culture, were described.

A protocol leading to reproducible propagation of *Rhizopus* sp. was reported.

A growth medium-dependent variation of *R. japonicus* morphology was reported. Such variations may be of taxonomic significance.

Aspects of the effects of growth conditions and medium composition on *R. javanicus* biomass propagation were reported. Illumination, shear and gas transfer all affected the mycelium.

It was discovered that in suspension culture *R. javanicus* biomass sequestered trace metals from the nutrient broth to such an extent that it limited its own growth.

The biosorbent (cell wall) composition (and subsequent metal uptake behaviour; see above) was found to be affected by the divalent cation content of the growth medium. This was related to the synthesis of cell wall polysaccharides containing ionizable groups.

7. Suggestions for Further Work

These studies have elucidated aspects of the physiology of Rhizopus in batch culture and have illustrated the possibility of changing the biosorbent characteristics by altering the cation content of the growth medium. Many interesting questions regarding the physiology of the organisms remain to be answered, and these will be addressed below. However, the time has come to apply biosorbents commercially, and this will be discussed first.

7.1 Application of Biosorbents

Although it is possible to grow biomass solely for use as a biosorbent, the economics of the process would doubtless be improved if waste biomass could be obtained. It is on this basis that the following suggestions are made.

1. Identify applications for biosorbents; ie. waste streams or process streams from which metals are to be concentrated.
2. Identify possible sources of waste biomass.
3. Investigate the possibility of modifying the medium and extending the time in the fermentor by a few hours to improve uptake capacity and/or selectivity.
4. Investigate harvesting, washing and processing of the biomass to produce biosorbent in a form which is easily transported and amenable to solution contacting. This would include drying and pelletization, if the organism is not already in such a form.
5. Investigate the behaviour of the biosorbent in terms of loading and breakthrough behaviour in packed and fluidized beds.
6. Define the operation to be used, either one-time metal contacting after which the biomass would be disposed of (possibly

smelted to recover valuable components) or repeated contacting with recycle of the solid, in which case the biosorbent would be utilized much as a conventional ion-exchange resin or adsorbent. In either case the treatment of the biomass after the initial metal-contacting period must be determined. The literature regarding biosorbent reuse should be reviewed in the context of particular applications.

7.2 Physiology of Rhizopus

1. Investigate the roles of anionic phosphate and uronic acid groups in stabilization of the wall structure. To what extent are they interchangeable, and to what extent does phosphate incorporation increase when poly-glucuronic acid synthesis is partially inhibited?

2. What is the function of the protein in the cell walls, and what differences in composition occur when the chitin/chitosan formation is partially inhibited?

3. Investigate the changes in cell wall composition and structure which occur during a short period of growth following stimulation of a stationary culture.

4. Investigate further the effects of growth rate on chitosan formation using other methods to vary the growth rate. The temperature of the culture is one possibility.

5. Investigate pelletization of the growing biomass with a view to propagating a biosorbent more amenable to commercial use. This may include the effect of ethanol in the medium on mould morphology, or the growth of the mould on a solid substrate.

6. Investigate the effect of acetate in the growth medium on chitin deacetylation, cell wall make-up and biosorbent quality.

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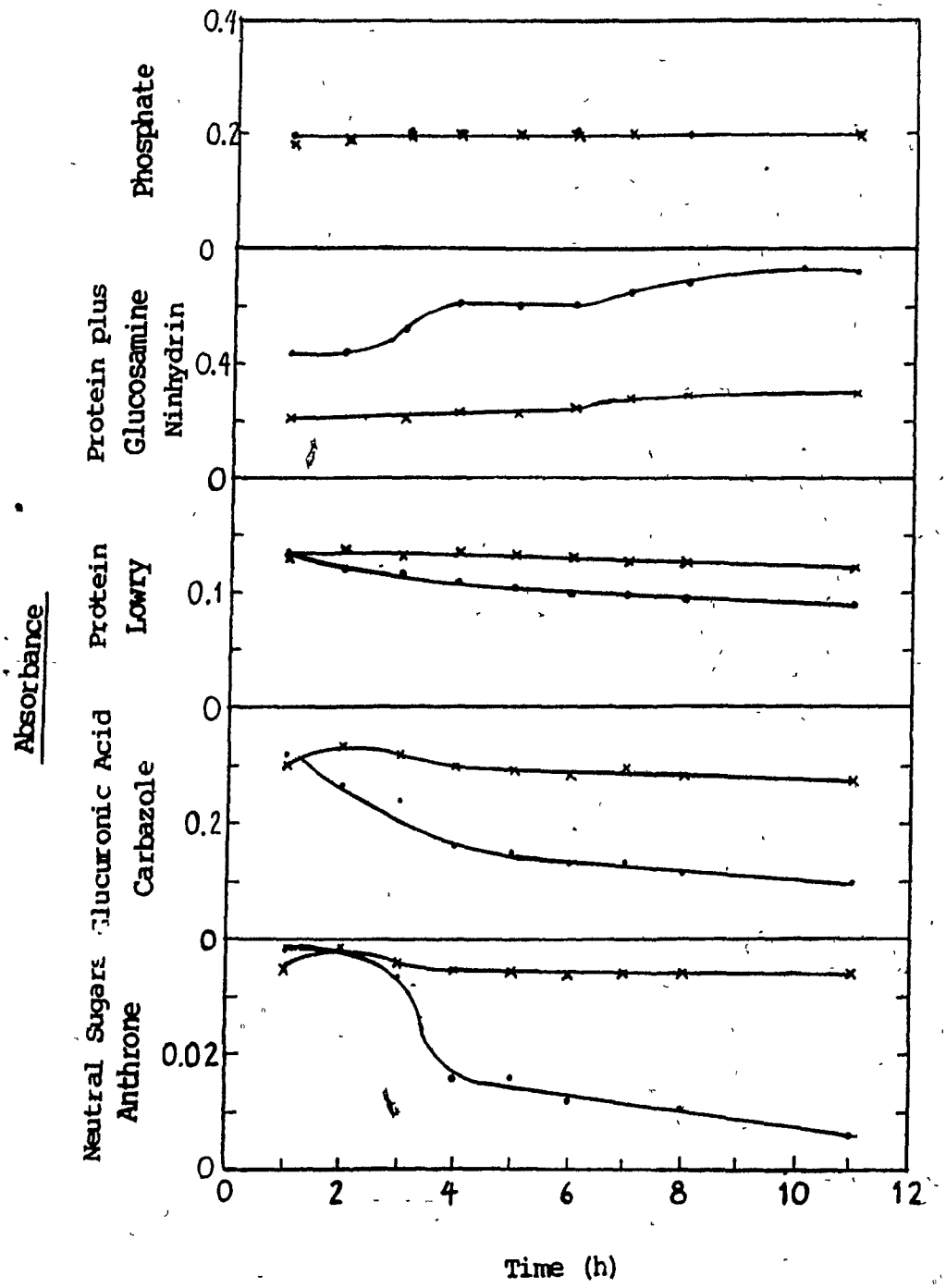
Appendix 1: Optimization of Biosorbent Hydrolysis Conditions Prior to Chemical Analysis

Prior to analysis of phosphate, protein, and neutral, acidic and amino sugars in biosorbent samples, these components had to be solubilized. This was accomplished by hydrolysis of 0.2 g of solid in 8.5 mL of 1.2 M or 6 M HCl at 100°C in sealed tubes. To determine the optimum times of hydrolysis, samples of the acid-washed old HSM biosorbent were treated for 1 to 11 h and assayed for the components (Figure A-1). Neutral and acidic sugars were liberated after two hours of mild hydrolysis in 1.2 M HCl, and subsequently underwent degradation. Harsh hydrolysis for ten hours in 6 M HCl was chosen for the preparation of samples for analysis of glucosamine and protein. Phosphate was liberated by both procedures and was assayed in both sets of samples, both in the preliminary experiments and in those reported in Section 3.4.1. These analyses were within 2% of one another, thus demonstrating that no serious errors occurred during manipulation of the samples.

Figure A-1: Time Study of Release of Acid-Washed Old HSM Biosorbent Components During Hydrolysis in 1.2 M and 6 M HCl

× 1.2 M HCl • 6 M HCl



Appendix 2: Non-linear Regression Program for Modelling of Metal Uptake Behaviour as Equilibrium with One, Two or Three Theoretical Sites on the Biosorbent

```
 15   Input"Data to come from file? (filename or n) ",C$
 16   If C$<>"n" And C$<>"N" Then Goto 4000
 20   Input"number of independent variables  ",M
 30   Input"number of measurements  ",O
 40   Dim Y(O,1),V1(O,M),V(M),Wt(O)
 50     For J=1 To O
 60     @ : @"experiment no. ";J
 70     Input"Y value  ",Y(J,1)
 75     Rem Y = uptake value
 80       For K=1 To M
 90       @"variable no.   ";K;
100       Input V1(J,K)
105       Rem V1 = metal concentration remaining in solution
110       Next K
120     Next J
130  @"expt no.       Y      independent variables(in order)"
135  @
140     For J=1 To O
150     Wt(J)=1/(Y(J,1)*Y(J,1))
160     @"   ";J;"          ";Y(J,1);"   ";
170       For K=1 To M
180       @"   ";V1(J,K);
190       Next K
200     @
210     Next J
220  @"Do you want to make any changes? ";
225  Input"Input row no.(0 to skip)  ",J
230  If J<1 Or J>O Then Goto 302
240  @"experiment no. ";J
250  Input"Y value  ",Y(J,1)
260     For K=1 To M
270     @"variable no.   ";K;
280     Input V1(J,K)
290     Next K
300  Goto 130
302  Input"Data to go to file? (Filename or n)  ",C$
303  If C$<>"n" And C$<>"N" Then Goto 4120
310  Input"number of terms in beta  ",N : Dim B(N)
311  Rem No. terms in Beta = twice the no. of theoretical sites
312  Rem odd B's = k*W    even B's = k
320     For J=1 To N
330     @"initial beta";J;
340     Input B(J)
350     Next J
```

```
480   Dim A(N,N),U(N,N),L(N,N),Z(N,N),Yl(N,N),Ai(N,N)
485   Dim X(O,N),Db(N),Xty(N),Dydb(N)
490     For Iter=1 To 20
500     Mat A=0 : Mat U=0 : Mat L=0 : Mat Z=0 : Mat Yl=0
505     Mat X=0 : Mat Xty=0
510     Ssq=0
520       For J=1 To O
530         For K=1 To M
540         V(K)=Vl(J,K)
550         Next K
560       Ey=V(1)*B(1)/(1+B(2)*V(1))
561       If N=2 Then Goto 570
562       Ey=Ey+V(1)*B(3)/(1+B(4)*V(1))
563       If N=4 Then Goto 570
564       Ey=Ey+V(1)*B(5)/(1+B(6)*V(1))
570       Y(J,0)=Y(J,1)-Ey
580       Ssq=Ssq+Wt(J)*Y(J,0)*Y(J,0)
585       Rem    Residual uptake weighted by division by
586       Rem    the solution conc'n
590       Next J
600     @ : @ Iter;" ";Ssq;" ";
610       For J=1 To N
620       @ B(J);" ";
630       Next J
640     @ : If Iter=1 Then Goto 740
650     If Abs(Ssq1-Ssq)/Ssq<1E-08 Then Goto 1970
660     If Ssq<Ssq1 Then Goto 740
670     Damp=Damp+1
680     If Damp>10 Then @"Process not converging." : Goto 2150
690       For J=1 To N
700       Db(J)=Db(J)/2
710       B(J)=B(J)-Db(J)
720       Next J
730     Goto 510
740     Damp=0
750       For J=1 To O
760         For K=1 To M
770         V(K)=Vl(J,K)
780         Next K
790       Dydb(1)=1/(1/V(1)+B(2))
800       Dydb(2)=-B(1)*Dydb(1)*Dydb(1)
805       If N=2 Then Goto 890
810       Dydb(3)=1/(1/V(1)+B(4))
820       Dydb(4)=-B(3)*Dydb(3)*Dydb(3)
821       If N=4 Then Goto 890
822       Dydb(5)=1/(1/V(1)+B(6))
823       Dydb(6)=-B(5)*Dydb(5)*Dydb(5)
890         For K=1 To N
900         X(J,K)=Dydb(K)
910         Next K
920       Next J
930     Rem calculate xTx
940     Mat Ai=0
950       For I=1 To N
960         For J=I To N
```

```
970           For K=1 To 0
980           A(I,J)=A(I,J)+Wt(K)*X(K,I)*X(K,J)
990           Next K
1000        A(J,I)=A(I,J)
1010        Next J
1020      Next I
1030      For J=1 To N
1040      A(J,0)=J
1050      Next J
1060    Row=1 : X1=Abs(A(1,1))
1070      For J=1 To N
1080      If Abs(A(J,1))>X1 Then Row=J : X1=Abs(A(J,1))
1090      Next J
1100    If Row<>1 Then K=1 : Gosub Switch
1110      For K=1 To N
1120      U(1,K)=A(1,K)
1130      Next K
1140      For I=2 To N
1150      L(I,1)=A(I,1)/U(1,1)
1160      Next I
1170      For K=2 To N
1180      If K=N Then Goto 1290
1190      X1=0
1200      Row=K
1210        For J=K To N
1220        Y2=A(J,K)
1230          For P=1 To K-1
1240          Y2=Y2-L(J,P)*U(P,K)
1250          Next P
1260        If Abs(Y2)>X1 Then X1=Abs(Y2) : Row=J
1270        Next J
1280      If Row<>K Then Gosub Switch
1290        For J=K To N
1300        U(K,J)=A(K,J)
1310          For P=1 To K-1
1320          U(K,J)=U(K,J)-L(K,P)*U(P,J)
1330          Next P
1340        Next J
1350      If K=N Then Goto 1440
1360      If Abs(U(K,K))<1E-50 Then Goto 2145
1370        For I=K+1 To N
1380        L(I,K)=A(I,K)
1390          For P=1 To K-1
1400          L(I,K)=L(I,K)-L(I,P)*U(P,K)
1410          Next P
1420        L(I,K)=L(I,K)/U(K,K)
1430        Next I
1440      Next K
1450    If Abs(U(N,N))<1E-50 Then Goto 2145
1460      For K=1 To N
1470      Z(K,K)=1/U(K,K) : Y1(K,K)=1
1480      Next K
1490      For J=2 To N
1500        For I=J-1 To 1 Step-1
1510          For K=I+1 To J
```

```
1520          Z(I,J)=Z(I,J)-U(I,K)*Z(K,J)
1530          Next K
1540        Z(I,J)=Z(I,J)/U(I,I)
1550        Next I
1560      Next J
1570      For J=1 To N-1
1580        For I=J+1 To N
1590          For K=J To I-1
1600          Y1(I,J)=Y1(I,J)-L(I,K)*Y1(K,J)
1610          Next K
1620        Next I
1630      Next J
1640      For J=1 To N
1650      Col=A(J,0)
1660        For I=1 To N
1670          For K=I To N
1680          Ai(I,Col)=Ai(I,Col)+Z(I,K)*Y1(K,J)
1690          Next K
1700        Next I
1710      Next J
1720      For J=1 To N
1730        For K=1 To O
1740        Xty(J)=Xty(J)+Wt(K)*Y(K,0)*X(K,J)
1750        Next K
1760      Next J
1770      For K=1 To N
1780      Db(K)=0
1790        For J=1 To N
1800        Db(K)=Db(K)+Xty(J)*Ai(J,K)
1810        Next J
1820      B(K)=B(K)+Db(K)
1830      Next K
1840    Ssq1=Ssq
1850    Next Iter
1860  @"Process has failed to converge." : Goto 2150
1870 *Switch
1880    For J=0 To N
1890    X1=A(K,J)
1900    A(K,J)=A(Row,J)
1910    A(Row,J)=X1
1920    X1=L(K,J)
1930    L(K,J)=L(Row,J)
1940    L(Row,J)=X1
1950    Next J
1960  Return
1970  @ : @"Process has converged. ";
1980  @"B values and standard deviations are:-" : @
1990    For J=1 To N
2000    @ J;"    ";B(J);"      +/-  ";Sqr(Ssq*Ai(J,J)/(O-N))
2010    Next J
2020  @ : @"Correlation coeffecient matrix:-" : @
2030  C$="--.###"
2040    For I=1 To N
2050      For J=1 To N
2060      @ Using C$,Ai(I,J)/Sqr(Ai(I,I)*Ai(J,J));"  ";
```

```
2070      Next J
2080    @
2090    Next I
2100  Stop
2110  @ : @"No.   Value       Residual"
2120    For I=1 To O
2130    @ I;"     ";Y(I,1);"     ";Y(I,0)
2140    Next I
2141  Goto 2150
2145  @"Matrix singular."
2150  Input"Do you want to try again? ",C$
2160  If C$="n" Or C$="N" Then Stop
2170  Input"Do you want to change the data? ",C$
2180  If C$="y" Or C$="Y" Then Goto 130
2190  Goto 310
4000  On Error Goto 15
4010  Open\1\C$
4015  On Error Stop
4020  Input\1\M : Input\1\O
4030  Dim Y(O,1),V1(O,M),V(M),Wt(O)
4040    For J=1 To O
4050    Input\1\Y(J,1)
4060      For K=1 To M
4070      Input\1\V1(J,K)
4080      Next K
4090    Next J
4100  Close
4110  Goto 130
4120  On Error Goto 4300
4125  Open\1\C$
4126  On Error Stop
4130  @\1\M : @\1\O
4140    For J=1 To O
4150    @\1\Y(J,1)
4160      For K=1 To M
4170      @\1\V1(J,K)
4180      Next K
4190    Next J
4200  Close
4210  Goto 310
4300  Create C$
4310  Goto 4125
```