Metal Accumulation by *Spirulina platensis* and its use in Biosorption Technology

THESIS
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To My Parents

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CERTIFICATE

This is to certify that the thesis entitled "**Metal Accumulation by *Spirulina platensis* and its use in Biosorption Technology**" submitted by **V. RAVEENDER, ID No. 2000PHXF010** for award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

SKVerma

Signature in full of the Supervisor

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Designation: **Assistant Professor & Group Leader**

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PREFACE

The Natural resources are exploited from time immortal for selfish human needs. The present endeavor *dares to exploit them further by using the tiny eco-friendly microorganisms* towards the *remediation of pollution that results from the ruthless voluntary or involuntary industrious-urban activities.* When the idea of present investigation was conceived during my first meeting with my Supervisor it made me to think scientifically and sentimentally to the present aspect of investigation that has led to nothing other than the present knowledge that I see with my own eyes.

The investigation was carried out with an objective of developing a suitable and 'herbal' alternative to the existing technologies for the removal and recovery of heavy metals including radionuclides, due to the increasing awareness of these toxicants when released into the environment. Just to simplify the complete investigation, the results are presented in five different sections.

In order to select the suitable strain, metal sensitivity, metal uptake ability, biomass stability under harsh environmental conditions and simpler nutrient requirements for cultivation of selected strain, are the key factors that stimulated us to look into cyanobacteria for preliminary screening. In first section of experimentation, the various types of cyanobacteria were screened for their metal sensitivity and uptake. Among the cyanobacteria, *Spirulina platensis* with moderate metal sensitivity and higher metal accumulation was selected for further studies. The second section deals with characterization of Co^{2+}, Cu^{2+} and Zn^{2+} uptake by normal *s.platensis* involving effect of pH, biomass concentration, metal concentration, kinetics, influence of light, temperature, co-ions, SEM, TEM and IR studies in order to know the basis of metal uptake, its localization and chemical nature. The third section focuses on comparing efficiency of lyophilized and oven-dried forms of biomass with normal cells for rigorous industrial applications involving effect of pH, metal concentration, kinetics, elution of metal, reuse of biomass over multiple cycles of sorption and elution, temperature and co-ions. Since, immobilization of the organism incorporates microbial biomass into an engineering process, the fourth section devoted to the immobilization of *S.platensis* using two techniques (Alginate and Acrylamide gel) and its recharacterisation for Co^{2+}, Cu^{2+} and Zn^{2+} uptake in terms of biomass loading in to gels, metal concentration, kinetics, elution of metal, reuse of immobilized biomass for multiple cycles of metal sorption and elution and co-ions. The last section of investigation deals with the continuous removal and recovery of Cu^{2+} and Zn^{2+} by a fixed-bed columnar reactor packed with polyacrylamide gel immobilized *S.platensis* biomass. This section also highlights the possible use of these techniques for treatment of electro-plating and nuclear industrial waste.

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List of Abbreviations

ATP	Adenosine Tri Phosphate
Ca-Alg	Calcium Alginate
CF	Concentration Factor
d	day (s)
DF	Decontamination Factor
Fe-EDTA	Iron-containing Ethylenediamine Tetra Acetic Acid
hr	Hour (s)
IR	Infra-Red
min	Minute (s)
Na_2-EDTA	Sodium-containing Ethylenediamine Tetra Acetic Acid
OD	Optical Density
PAG	PolyAcrylamide gel
rpm	Revolutions per Minute
RT	Retention Time
SEM	Scanning Electron Microscope
TEM	Transmission Electron microscope
v/v	Volume by Volume ratio
VRF	Volume Reduction Factor
w/v	Weight by Volume ratio

Introduction

Chapter I
INTRODUCTION

Metallic species mobilized and released into the environment by the anthropogenic activities tend to persist indefinitely by circulating and eventually accumulating throughout the food chain, posing a serious threat to the biota and environmental sustainability (Forstner and Wittmann 1979, Volesky and Holan 1995, Nies 1999, Gadd 2000). The invention and the development of new technologies involving nuclear fission opened up a whole new area of hope and concern at the same time. Radioactive isotopes of elements have been discovered and handled in historically unprecedented quantities and concentrations. The danger associated with radioactivity dissipation particularly with long-lived and high-radiation isotopes is cause of major concern (Schmidt and Moffett 1979). The removal of metal ions including radionuclides from the environment has become an important priority in radioactive and non-radioactive industrial waste management, which is reflected in the stricter enforcement of environmental regulations (Davis *et al.* 2000). The classical strategies are oriented towards either precipitation or the concentration of contaminants followed by their immobilization into suitable matrix leading to compartmentalization of the metals to a part of the environment in which their harm is reduced (Barkay and Schaefer 2001). While conventional technologies either do not remove trace metals completely or they are too costly for implementation, biosorption appears to offer a technically feasible and economically attractive alternative (Macaskie *et al.* 2000, Yalcinkaya *et al.* 2002). As a result an understanding of microbe-based partitioning of metals between gaseous, solid

and liquid phases *in situ* is being investigated as a scientific basis for metal and radionuclide bioremediation (Barkay and Schaefer 2001).

Heavy metals are metals having a density above 5 g cm^{-3} (Weast 1984). Although most heavy metals are toxic to microorganisms beyond threshold concentrations, some of them like Cu, Zn and Mn are required in trace quantities for various physiological and biochemical processes whereas others have no known biological function. In general, heavy metals are known to increase the generation time of algae and other microorganisms, leading to the metal-induced toxicity (De Filippis and Pallaghy 1976, Conway 1978, Stratton *et al.* 1979, Rai *et al.* 1981). The primary target of heavy metals is shown to be the cell membrane as observed in the change in metal-induced membrane potential or loss of electrolytes (Passow and Rothstein 1960). The effects of heavy metals on microbes in general include blockade of sulfhydryl group (Bonaly *et al.* 1980), reduction of ATP generation (Stratton *et al.* 1979), photosynthetic oxygen evolution (Verma and Singh 1991) and nitrogen fixation and assimilation (Rai and Raizada 1986). Thus, if the use of living cells is envisaged in a metal removal system, the metal toxicity leading to the inactivation of the biomass (Benemann and Wilde 1991) becomes a bottleneck in any microbe based metal removal technology. Microbes encounter metals of various kinds in their ambient environment and it is therefore, not surprising that they have developed various mechanisms to counteract these toxicants (Ehrlich 1997). Microorganisms, including actinomycetes, bacteria, cyanobacteria, algae, fungi, and yeasts have developed the ability to accumulate and detoxify heavy metals and radionuclides from their external environment (Trevors *et al.* 1986, Luef *et al.* 1991, Matsunaga *et al.* 1999). The microbial response to the metals varies from physico-chemical interactions like adsorption and deposition on cell wall to the processes

dependent on cell metabolism, e.g., transport, internal compartmentalization and extra cellular precipitation (Kotrba *et al.* 1999, Gutnick and Bach 2000). In addition, tolerance and resistance are widely found properties of microorganisms of all the major groups (Gadd and Griffiths 1978, Trevors *et al.* 1986). Development of these mechanisms, by microbes, could be attributed owing to their shorter generation time and consequent upon their higher rate of evolution (Wood and Wang 1983). These properties, however, have given rise to considerable interest in the use of microbial biomass to remove metals and radionuclides from industrial wastes. In certain cases, resistant strains of algae are reported to take up less metal ions than the sensitive strains. This occurs in copper-tolerant strains of *Chlorella vulgaris,* which operate a cellular extrusion mechanism (Butler *et al.* 1980). However, most resistant strains accumulate more metal than their sensitive strains. For example a copper and nickel - tolerant strain of *Scenedesmus acutiformis* was found to take up several thousand μg Cu per g dry wt. without affecting its viability whereas a sensitive strain died at 650 μg g^{-1} (Stokes 1983).

The use of dead biomass or derived products with a number of advantages over living cells, have been shown to perform better than the conventional waste water treatment processes (e.g., chemical precipitation, ion-exchange, membrane filtration or electrochemical separation) in being cost-effective, minimizing the chemical/biological sludge volume, high efficiency even with very dilute effluents and in developing the non-destructive elution techniques (Tsezos *et al.* 1995, Kratochvil and Volesky 1998).

Microbial metal uptake is divided into two main phases; the first phase common in living and dead cells, is metabolism-independent binding or adsorption to cell exterior is rapid and reversible, followed by a second slower phase of metabolism-dependent intracellular uptake (Verma and Singh 1990, Corder and Reeves 1994). The relative

contribution of these two phases in the metal uptake process varies with the type of metal and the microbe involved, e.g., surface accumulation contributes the major share in lead, uranium, and thorium accumulation by living or dead cells. Metabolism-independent accumulation phase is unaffected by metabolic inhibitors, modest alteration of temperature, uncouplers, and light/dark cycles (Trevors *et al.* 1986, Volesky and Schiewer 1999). In living cells, any one or both these phases of uptake may be influenced by change in the chemical nature of the growth medium, and excretion of substances that may complex or precipitate metals. The faster intracellular uptake during the first phase by passive diffusion could also result due to the increased permeability of membranes in response to heavy metals. For example, the active uptake of metals by algae is shown to be sensitive to light, uncouplers, metabolic inhibitors, and temperature (Skowronski 1986, Verma and Singh 1990, Verma and Singh 1991). Both these process may lead to a cellular metal accumulation, which is several thousand fold greater than the external concentration (Wood and Wang 1984).

The key factors in the selection of biomass for metal biosorption technology are the reaction mechanisms and the process employed for metal recovery. The non-destructive process of metal recovery may support repeated cycling of biomass whereas destructive process can not allow the reuse of biomass over multiple cycles (Gadd 1988). The choice of a recovery process also depends on the mechanism of accumulation. Metabolism-independent biosorption is often reversible and can support non-destructive desorption whereas metabolism-dependent intracellular accumulation is often irreversible, requiring destructive recovery of absorbed metal (Volesky 1990). In most of the studies, non-destructive desorption from loaded biomass have been employed (Gadd 1988).

Biosorption is defined as sequestration of metal ions by materials of biological origin. It must be distinguished from bioaccumulation, which is usually an active and metabolically mediated process found in living organisms (Ashley and Roach 1990, Volesky and Schiewer 1999). The term biosorption refers to several modes of metal uptake by biomass, which may be live or dead (Figueira *et al.* 2000). Metal sequestration by different parts of the cell can occur via complexation, coordination, chelation, ion exchange, adsorption, inorganic microprecipitation (Schiewer and Volesky 2000). Any one or a combination of the above metal-binding mechanisms may be functional to various degrees in immobilizing one or more metallic species on the biosorbents (Matheickal *et al.* 1997). Metallic cations are attracted to negatively charged sites at the surface of cell, thereby a number of anionic ligands i.e. phosphoryl, carboxyl, sulfhydryl, amide and hydroxyl groups of membrane proteins participate in metal binding (Small *et al.* 1999, Texier *et al.* 2000). Langley and Beveridge (1999) showed that metals bind most likely to phosphoryl groups in the core-lipid A of lipopolysaccharide, and the negatively charged side chains influence their binding to gram-negative bacteria by affecting cell hydrophobicity.

The Adsorption of Cd^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} is generally decreased at low pH in a variety of algae. However, there are reports of strong binding of Au^{3+} and Hg^{2+} at pH 2.0 in *C. vulgaris* (Darnall 1986). The biosorption of heavy metals and radionuclides by a variety of freshwater and marine algae have been demonstrated with Freundlich and/or Langmuir isotherms confirming a linear equilibrium relationship between the metal present in solution and bound to the cell surface (Khummongkol *et al.* 1982). Another parameter known to affect biosorption is cell density, which shows a general trend of decrease in specific metal uptake with increasing biomass concentration (Sakaguchi *et al.*

1979). Biosorption can be affected by the presence of other ions, as Ca^{2+}, Mg^{2+}, Na^{+}, Mn^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+}, but not K^{+}, have been shown to reduce Cd^{2+} binding by *Chlorella regularis* (Sakaguchi *et al.* 1979). While there are several reports on biosorption of one metal from solution containing several metal ions (Mallick and Rai 1994, Chong and Volesky 1996, Figueira *et al.* 1997), a little attention is paid to simultaneous sorption of several metals from multi metallic systems (Mehta and Gaur 2001).

The mobility of the biomass, irrespective of its viability, used in biosorption process is an important consideration for a metal recovery system. While cells in free suspension can provide vital information under laboratory conditions, the same may have several disadvantages during scale-up process. The small particle size, low mechanical strength, and low density can limit the choice of reactor systems and make biomass separation difficult (Tsezos 1986). The use of immobilized or pelleted biomass in packed-bed or fluidized-bed reactors has shown greater potential owing to its reusability, easy separation from the reaction mixture in high continuous flow systems. In this technique, biomass particle size can be controlled and high flow rates of influent can be tolerated with or without their recirculation (Scott 1987). For immobilization, gel entrapment retains cell suspension in a bioreactor for high-density operations without the difficulties of cell separation. The primary advantage of gel entrapment elucidated in earlier studies is, that it allows a stirred tank or airlift bioreactor to be operated as perfusion system for suspension cell lines by simply installing a screen over the influent that removes the cell free effluent (Seifert and Phillips 1997). Various synthetic matrices used in immobilizing the microbial biomass are calcium alginate (Singh *et al.* 1989, White and Gadd 1990), silica (Feiler and Darnell 1991), polyacrylamide (Brady and Duncan

1994), polyurethane (Hu and Reeves 1997), polysulfone (Seidel and Jeffers 1991, Trujillo *et al.* 1991), epichlorhydrin (Fogarty *et al.* 1999), etc.

The efficacy of a metal treatment process is evaluated by the values of decontamination factor (DF), which is defined as the 'ratio of metal concentration in the liquid phase, before and after the treatment'. In addition to obtaining high DF values, the process should also yield high volume reduction factor (VRF), which is defined as the 'ratio of the initial volume of the waste to the volume generated after treatment'. Therefore, the volume reduction is an essential and obligatory step towards the 'Efficient Waste Management' strategy (Sinha and Ahmed 1997). To achieve high DF and high VRF, the metal absorbed by the biomass has to be followed by an efficient release of metal in concentrated form (Wood and Wang 1983). This part of the process, similar to ion-exchange process, is based on eluting the metal by a small volume of eluant, which would ideally regenerate the biosorbent for a subsequent cycle. In general, the eluant uncouples the bonds sequestering the metal from the biosorbent surface without damaging its chemical structure, thus making it reusable in multiple uptake/elution cycles (Volesky 1990). The solid to liquid ratio (S/L), which represents the ratio between the sorbent and eluant, is often used to determine the efficiency of the elution process. High values of S/L are desirable for preferably complete elutions (Gadd 1988), but it is the overall metal specificity of the combined uptake-elution process, which is of ultimate technological significance for repeated cycles. Waste biomass applied as a biosorbents may, in some cases, be so cheap and abundant that its recycling may not be worthwhile and its combustion would yield ash with a high concentration of the desired metal. This approach, of course, would have to be based on overall process-feasibility considerations. It may be of particular significance where the biosorbents would consist

of either a raw biological waste material from other industrial processes (e.g., industrial fermentations) or as a naturally abundant renewable biomaterial (algae) that can be cheaply harvested (Gadd 1988).

The process of metal recovery using microbial biosorbent materials is basically a solid-liquid contact process consisting of metal uptake (sequestering) and metal desorption (elution) cycles. Its technological configuration is very similar to that used in the ion-exchange process or activated carbon applications. The metal-laden solution is in contact with the solid sorbent phase in a batch, semi-continuous, or continuous-flow arrangement. Appropriate contact between the solution and the solid phase can be accomplished in a variety of bioreactors. Localizing the metal deposition site within the biosorbents and understanding the metal-sequestering mechanism, in combination with elucidation of the relevant metal solution chemistry and chemical structure of the metal deposition site, are all crucial aspects of the quest for an efficient biosorption process which should feature high metal selectivity and uptake (Volesky 1990).

The fixed packed-bed contactor has a column arrangement where the biosorbent granules are packed into a solid bed, which does not normally move. The liquid usually flows through the bed in an up-flow arrangement (Kogej and Pavko 2001). The fixed bed continuous flow contact system is an application oriented approach, allowing more efficient utilization of the sorbent capacity and which is quite different from the batch biosorption system as the later is more useful in developing equilibrium sorption data (Volesky and Prasetyo 1994). Granules of the biosorbent have to be large enough (1 to 3 mm) to avoid an excessive pressure drop across the bed. However, too large a particle size would tend to decrease the operational surface area of the biosorbents, making the process feasibility limited by the intraparticle diffusion rate.

In certain environments and for specific applications, the use of algae as biosorbents has been a regular target of investigation. One such commercial product, called BIO-FIX (Darnall 1986) is a bead shaped resin containing biomass blended from *Sphagnum* and Algae. Other such products are B.V. Sorbes, Inc. (Kuyucak and Volesky 1989b), and biological ion-exchange resin (Jeffers *et al.* 1991) consists of non-living biomass (primarily algae) immobilized in a polymeric matrix. Each of these products has demonstrated its efficacy in removing heavy metal ions from solutions and its satisfactory reuse through numerous loading-elution cycles.

The present study was undertaken with an objective to identify a better biosorbent and its possible use in a continuous column reactor for the removal and recovery of heavy metals as well as radionuclides. In order to achieve this objective, the cyanobacteria were chosen as a suitable candidates as they are known for their adaptation to the stress (Borbely *et al.* 1990, Belkin and Baussiba 1991, Singh and Kumar 1994), stability during processing into biosorbent material and easy cultivation (Corder and Reeves 1994).

Cyanobacteria are a diverse group of prokaryotes, exhibiting versatile physiology and wide ecological tolerance that contribute to their competitive success over a broad spectrum of environments (Shilo 1989) including Antarctic dry valleys, cold (Friedmann 1982) and hot deserts (Potts and Friedmann 1981), tropical rain forest and mangrove swamps (Olson *et al.* 1990, Pawlik and Skowronski 1994), microplankton (Fogg 1982) and pikoplankton (Guillard *et al.* 1985). These oxygen-evolving organisms quickly respond and adapt to stress conditions in general (Slotton *et al.* 1989, Borbely *et al.* 1990) and heavy metals in particular (Gothalwal and Bisen 1993, Bender *et al.* 1994, Kanamaru *et al.* 1994). They have developed natural methods of resistance towards heavy metals, viz., a reduction in metal intake (Singh and Yadava 1986), extracellular sequestration

(Silver and Misra 1988), localization/compartmentalization inside the cell (Jensen *et al.* 1982, Zhang and Majidi 1994) or energy-dependent efflux (Verma and Singh 1991), etc. Considering these features, the present investigation is undertaken with the following objectives:

1. to identify the suitable microbe which can tolerate elevated metal concentrations,
2. to characterize the biosorbent which can accumulate higher concentration of metal ions generally abundant in industrial effluents, and
3. to develop a suitable technique for continuous metal removal and recovery using biosorbent-packed bed columnar reactor

Materials and methods

Chapter II
MATERIALS AND METHODS

1. Strains and culture conditions

Six cyanobacterial strains, viz., *Anabeana cylindrica* 847, *Aphanothece* sp. 782, *Aulosira fertilisma* 573, *Nostoc calcicola* 263, *Nostoc muscorum* 554 and *Spirulina pl atensis* 730, were procured from National Facility for Blue Green Algae (NFBGA), New Delhi (INDIA). Axenic cultures were established and maintained in growth medium mentioned below under culture room conditions illuminated with cool day light fluorescent tubes (14.4 Wm^{-2}). The composition of different media is mentioned in Table-1.

Cyanobacteria	*Media used*
A. cylindrica	Fogg's (w/o nitrate) (Fogg 1987)
Aphanothece sp.	Fogg's (with nitrate) (Fogg 1987)
A.fertilisma	Fogg's (w/o nitrate) (Fogg 1987)
N.calcicola	Allen and Arnon (w/o nitrate) (Allen and Aronon 1955)
N.muscorum	Modified Chu-10 (w/o nitrate) (Gerloff *et al.* 1950)
S.platensis	Ogawa and Terui (Ogawa and Terui 1970)

2. Growth determination

To determine the growth pattern of cyanobacteria, the exponentially grown cells (6-d) were inoculated into flasks containing 200 mL growth medium (OD_{560} = 0.08) in 500 mL Erlenmeyer flasks and incubated at 26 ± 2 ^{0}C on a rotary shaker (180 rpm) under culture room conditions. The growth rate was determined by monitoring optical density and cellular protein content of the culture at a regular interval of 12 hr for a period of 0-8 days. Specific growth rate constant ($\underline{k}$) was calculated as described by Kratz and Myers (1955).

Table-1. Media used for maintenance of cyanobacteria.

Component	*Modified Chu 10 (g L^{-1})*	*Allen and Arnon (g L^{-1})*	*Fogg's (g L^{-1})*	*Ogawa and Terui (g L^{-1})*
$MgSO_4.7H_2O$	0.025	-	0.2	1.2
$Ca(NO_3)_2.4H_2O$	0.232	-	-	-
$Na_2SiO_3.5H_2O$	0.044	-	-	-
Na_2CO_3	0.020	-	-	-
K_2HPO_4	0.010	0.348	-	0.5
Ferric Citrate	0.0035	-	-	-
Citric acid	0.0035	-	-	-
$CaCl_2.2H_2O$	0.0735	-	0.1	0.04
$MgSO_4$	-	0.247	-	-
NaCl	-	0.230	-	1.0
$CaCl_2$	-	0.055	-	-
Micronutrients	a*	a*	-	-
Fe-EDTA	-	b#	b#	-
KNO_3	-	-	0.25	-
A_5 Solution	-	-	a^5	-
Na_2HCO_3	-	-	-	4.5[c]
$NaNO_3$	-	-	-	1.5
K_2SO_4	-	-	-	1.0
$FeSO_4$	-	-	-	0.1
KH_2PO_4	-	-	0.2	-
pH adjusted before autoclaving to	-	-	-	9.0

*a Micronutrients as mentioned in Table-2.

#b Fe-EDTA was prepared by dissolving 5.2 g of EDTA and 5.4 g $KHCO_3$ in 100 mL of distilled water followed by boiling to remove the CO_2. To the CO_2 free solution, 5 g of $FeSO_4$ was added, boiled. The solution was made up to 1 L after cooling it at room temperature. 1 mL of this stock was added to 1L of culture medium.

[c] Autoclaved separately and added to the culture medium after cooling.

Table-2. Composition of micronutrients.

Component	*Micronutrient for modified Chu 10 and Allen & Arnon medium (mg L^{-1})*	*Micronutrient (A_5) for Fogg's medium (g L^{-1})*
H_3BO_3	0.5	2.86
$ZnSO_4.H_2O$	0.05	--
$ZnSO_4.7H_2O$	--	0.222
$MnCl_2.4H_2O$	0.05	1.81
$CuSO_4.5H_2O$	0.05	0.079
MoO_3	0.01	0.0177
$CoCl_2$	0.04	--

3. Chemicals

All the salts, i.e. $MnCl_2$, $FeCl_3$, $Ni(NO_3).6H_2O$, $Co(NO_3).6H_2O$, $Cu(NO_3).3H_2O$, $Zn(NO_3).6H_2O$, $Cd(NO_3).4H_2O$, CsCl and $SrCl_2.6H_2O$, used in the preparation of metal solutions were of analytical grade (Merck, Germany). They were dissolved in deionized distilled water and digested using 1-2% nitric acid. The radioisotopes were obtained from Board of Radiation and Isotope Technology (BRIT), Mumbai in the form of $^{65}ZnCl_2$ and $^{85+89}Sr(NO_3)_2$ (specific activity 8.4 mCi g^{-1} and 7.8 Ci g^{-1} respectively). The solutions having radionuclides were prepared by spiking ^{65}Zn and $^{85+89}Sr$ separately. Atomic spectral standards (1000 mg L^{-1}) were procured from National Physical Laboratory (NPL), New Delhi. They were used to prepare working standards for all metals used in the Atomic Absorption study. Metal solutions and working standards were prepared afresh for every experiment by diluting the stock solutions. To eliminate contamination, all glassware and plasticware were cleaned before use with cedepol detergent, followed by an overnight soaking in 50% nitric acid.

4. Preparation of Biomass suspension

Biomass was harvested from the culture by centrifugation of late exponential phase cells (OD_{560} = 1.8 ≡ 0.99 mg mL^{-1} dry wt.) at 5000 rpm for 10 min using REMI COMPU*fuge* CPR 24. Harvested cells were washed twice with sterile deionised distilled water and were resuspended in deionised distilled water for use in metal uptake experiments involving normal biomass. Biomass concentration in cell suspension was determined by drying an aliquot in a pre-weighed glass mini container to constant weight at 80 °C. For lyophilizing biomass, the biomass was harvested by centrifugation of late exponential phase cells as described earlier followed by pre freezing at –20°C for 10 min before

freeze-drying at -110°C till wet biomass was concentrated to dry, fine and free flowing cells in the powder form using *Heto Maxi Iyo* (Germany). For metal uptake experiments involving dead biomass, harvested biomass after washing was oven dried at 80°C to constant weight before its use.

5. Determination of metal toxicity

The exponentially grown cyanobacterial cultures were centrifuged at 5000 rpm and washed thrice with sterile deionised distilled water. Such cells were inoculated into 50 mL of fresh growth medium (containing different metal ions with varying concentrations of 0.25-2000 mg L^{-1}) in 150 mL Erlenmeyer flasks with final cell density of 200 µg protein mL^{-1} (OD_{650} = 0.1) containing) separately. This was followed by phototrophic incubation of cells and regular monitoring of growth by measuring optical density of culture at 650 nm and cellular protein content for 8 days. The concentration, at which the optical density at 650nm and/or the total protein content of the cyanobacterial culture became almost zero, was taken as the toxic concentration of the target metal towards the organism.

6. Protein estimations

Protein content of cyanobacterial cultures was estimated by the method of Lowry *et al.* (1951), modified by Herbert *et al.* (1971).

The reagents used were,

Reagent A - 1.0 N NaOH

Reagent B - (i) 5.0% Na_2CO_3

(ii) 0.5% $CuSO_4.5H_2O$ in 1.0% sodium potassium tartrate

Solution (i) and (ii) were mixed in the ratio 25:1 (v/v)

Reagent C - 1.0 N Folin-phenol reagent

A 0.5 mL of Reagent A was added to 0.5 mL of cyanobacterial sample and kept in boiling water bath for 5 min followed by cooling under running water. 2.5 mL of Reagent B was added to it and incubated for 10 min at room temperature, followed by addition of 0.5 mL of Reagent C. The intensity of blue color developed after 15 min of incubation at room temperature was measured at 650 nm in the Spectrophotometer (JASCO, UV/VIS). The concentration of protein was estimated from a calibration curve prepared using Lysozyme (Sigma, USA) as standard and expressed as μg protein mL^{-1} of cyanobacterial culture.

7. Metal ion analysis

Metal ion concentrations were determined in a Perkin Elmer (*A Analyst 300*) atomic absorption spectrophotometer using air-acetylene or nitrous oxide-acetylene flames. All instrumental conditions were optimized for maximum sensitivity as described by the manufacturer. The instrument was first calibrated with respective metal standard solutions in the range covering the concentrations likely to be found in samples. The samples were then analyzed in triplicate to maintain good reproducibility. In addition, procedural blanks were also analyzed to check for contamination.

8. Quantification of metal uptake

The metal uptake was determined by the simple concentration difference method. While, the initial and final metal concentrations, Ci and Cf, respectively, were ($mg L^{-1}$) determined by Atomic Absorption Spectrophotometer, the metal ion uptake, q, was ($mg g^{-1}$) calculated from the mass balance as follows:

$$Q = (C_i - C_f)\, V/W \quad \text{.....Eq-1}$$

Where V is the solution volume (L) and W is the mass of biomass (g) (dry wt.). All the results on metal uptake were expressed on a dry-weight biomass basis. All data points in the

figures presented in this study are the mean of triplicate experimental results, and the deviations were found to be well within 5%.

9. Measurement of radioactivity

The radioactive metal concentrations present in the influent and effluent of packed bed columnar reactor were measured radiometrically using a well-type NaI (Tl) crystal scintillation probe (BICRON, USA) coupled to a single-channel analyzer (*pea* Gammaray Spectrometer, GRS-101P, INDIA). The 1.1 MeV and 0.51 MeV photo peak of ^{65}Zn ($t_{1/2}$ = 244d) and ^{85}Sr ($t_{1/2}$ = 65d) were used for the measurements of zinc and strontium activities.

10. Metal availability experiments

To test the effect of pH on metal availability, the pH of solutions containing desired concentration of different metals were adjusted to required values by the addition of 0.1 N nitric acid or 0.1 N Sodium hydroxide. After 24 hours of incubation, the solution was decanted and assayed for metal concentration by using the Atomic Absorption Spectrophotometer as described earlier.

11. Uptake experiments

The cyanobacterial cells in their exponential phase were collected as described earlier. The biomass was used directly or under lyophilized or oven-dried condition for incubation with metal solutions buffered with acetate buffering system (0.5 M CH_3COOH/CH_3COONa). The concentration of metal varied in different experiments and is described at the appropriate place. The uptake mixture was incubated under culture room conditions with continuous shaking on an orbital shaker at 180 rpm. After 2 hr of contact, the solutions were vaccum-filtered through 0.45 µm cellulose membrane filters as reported earlier (Raveender *et al.* 2002). Metal concentrations in the filtrate

were determined by Atomic Absorption Spectrophotometer (*A Analyst 300*, Perkin Elmer) as mentioned earlier. Each sample was analyzed in triplicate against procedural blanks.

11.1. Effect of pH

In order to test the effect of pH on metal uptake, the cell suspensions involving living, lyophilized or oven-dried biomass with the final concentration of 1.0 mg mL^{-1} was exposed to 100 mg L^{-1} of Co^{2+}, Cu^{2+} or Zn^{2+} solution. Metal solutions were adjusted to desired pH using 0.1 N nitric acid or 0.1 N sodium hydroxide before biomass addition. After required contact period, the samples were filtered using 0.45 μm membrane filters and the filtrate was assayed for residual metal concentrations.

11.2. Effect of biomass density

The effect of biomass density was checked by exposing different quantities of biomass (ranging from 0.5 mg mL^{-1} to 6 mg mL^{-1}) to the target metal solution containing 100 mg L^{-1} of Co^{2+}, Cu^{2+} or Zn^{2+} for 2 hr. Samples were filtered by using 0.45 μm membrane filters and assayed for residual metal concentration by Atomic Absorption Spectrophotometer.

11.3. Effect of metal concentration

For studying the effect of different metal concentrations, the biomass (1 mg mL^{-1}) was exposed to the solutions containing varying concentrations (50-800 mg L^{-1}) of Co^{2+}, Cu^{2+} or Zn^{2+}. The values of uptake obtained for different concentrations of metal were further used to study the sorption phenomenon using Langmuir (Langmuir 1918) or Freundlich (Freundlich 1926) equation of adsorption isotherms.

11.4. Linearised sorption isotherms

The performance of the biosorbent material is also evaluated in terms of its metal loading capacity (q) for a range of residual metal concentration. Towards better understanding of linearised isotherms, the single-sorbate sorption is best compared on a complete sorption isotherm curve derived under the same environmental conditions (e.g., pH, temperature, ionic strength etc.). In principle, the sorption isotherms are plots of the sorption (q) against the final concentration (C_e) of the sorbate remaining in the solution. The classical models of Langmuir (Eq-2) or Freundlich (Eq-3) describe such relationships as,

$$q = q_{max}\, bC_e/1+bC_e \quad \text{.....Eq-2}$$

$$q = kC_e^{1/n} \quad \text{.....Eq-3}$$

In Langmuir model, the constant 'q_{max}'(mg g^{-1}) is the maximum specific metal uptake and 'b'(L mg^{-1}) the ratio of the adsorption/desorption rates related to energy of adsorption through Arrhenius equation. In Freundlich equation, the constant 'k' is a measure of adsorption capacity and '1/n', the intensity of adsorption. For fitting the experimental data, both models were linearised as follows,

Langmuir Equation $1/q = 1/q_{max}\, b\, (1/C_e) + 1/q_{max}$ Eq-4

Freundlich Equation $\text{Ln } q = \text{Ln } k + 1/n \text{ Ln } C_e$ Eq-5

In all above-mentioned cases for the total of three metals with two forms of *S.platensis* biomass, such linearised models were examined and respective constants were derived.

11.5. Kinetics of metal uptake

To examine the metal uptake kinetics, the cells are suspended in metal solution with the final concentration of 1.0 mg mL^{-1} and incubated under culture room conditions as

described earlier. The samples were withdrawn at the desired time intervals and were subsequently filtered and assayed for residual metal concentration.

11.6. Effect of photosynthetic light and temperature

In order to examine the effect of photosynthetic light on metal uptake, the reaction vessels were incubated in dark and light conditions (14.4 Wm^{-2} illumination) for 2 hr followed by collection of sample and its analysis. For studying the effect of temperature, reaction vessels were incubated at different temperatures ranging from 5ºC to 50ºC followed by filtration of reaction mixture. Filtrates were analysed for residual metal concentration.

11.7. Effect of co-ions

The effect of co-ions on metal uptake by free cells of *S.platensis* was tested in binary metal systems. Biomass (100 mg, dry weight) was exposed to 100 mL binary metal solutions containing equimolar concentrations of Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+} and Cd^{2+}. The effect of cations on immobilized *S.platensis* biomass was conducted by incubating biomass in presence of increasing concentrations of co-ions while target ion concentration was kept constant (at its saturation level) in all the cases. After the required incubation period samples of metal solutions were withdrawn and filtered for further analysis as mentioned earlier.

12. Immobilization

12.1. Immobilization in calcium alginate (Ca-Alg) matrix

The late exponential phase cyanobacterial cells (equivalent to 0.99 mg dry weight mL^{-1} culture) obtained by centrifugation and repeated washings, were either directly or after lyophilyzation, suspended in 1.5% (w/v) aqueous solution of sodium alginate (150 mL) and was injected through a hypodermic syringe into magnetically stirred 0.2 M calcium

chloride solution (500 mL) as described by Singh *et al.* (1989). The spherical beads (2-mm diameter; Plate 1a) of calcium alginate thus formed were allowed to harden for 24 hr before washing them with sterile distilled water. Control beads were prepared using same procedure without biomass.

12.2. Immobilization in polyacrylamide gel (PAG) matrix

The immobilization of biomass in the polyacrylamide gel matrix (PAG) was carried out as described by Tucker *et al.* (1998). The late exponential phase cyanobacterial cells (equivalent to 0.99 mg dry weight mL^{-1} culture) obtained by centrifugation and repeated washings, were, suspended in 20 mL sterile water either directly or after lyophilyzation. An aliquot of this cell suspension was also collected and dried at 105 ^{0}C for determination of cell dry weight. The suspension was supplemented with 2.5 g acrylamide monomer and 0.25 g N,N'- methylenebisacrylamide as a cross-linking agent. The polymerizing reaction was initiated by adding 2.5 mL of a 2.5% (w/v) solution of potassium persulphate, and accelerated by adding 2.5 mL of a 5% (w/v) solution of 3-dimethylaminopropionitrile. The suspension was shaken gently until the gel began to set and was then refrigerated at $4^{0}C$ throughout polymerization to prevent thermal damage to the cells. The gels were prepared in a closed rectangular tray, made up of acrylic sheet, having openings at two sides. When the gel had set, these two openings were unlocked and the gel was cut in to 9 mm^3 cubes (Plate 1b). Cell free cubes were also prepared following the similar procedure described above.

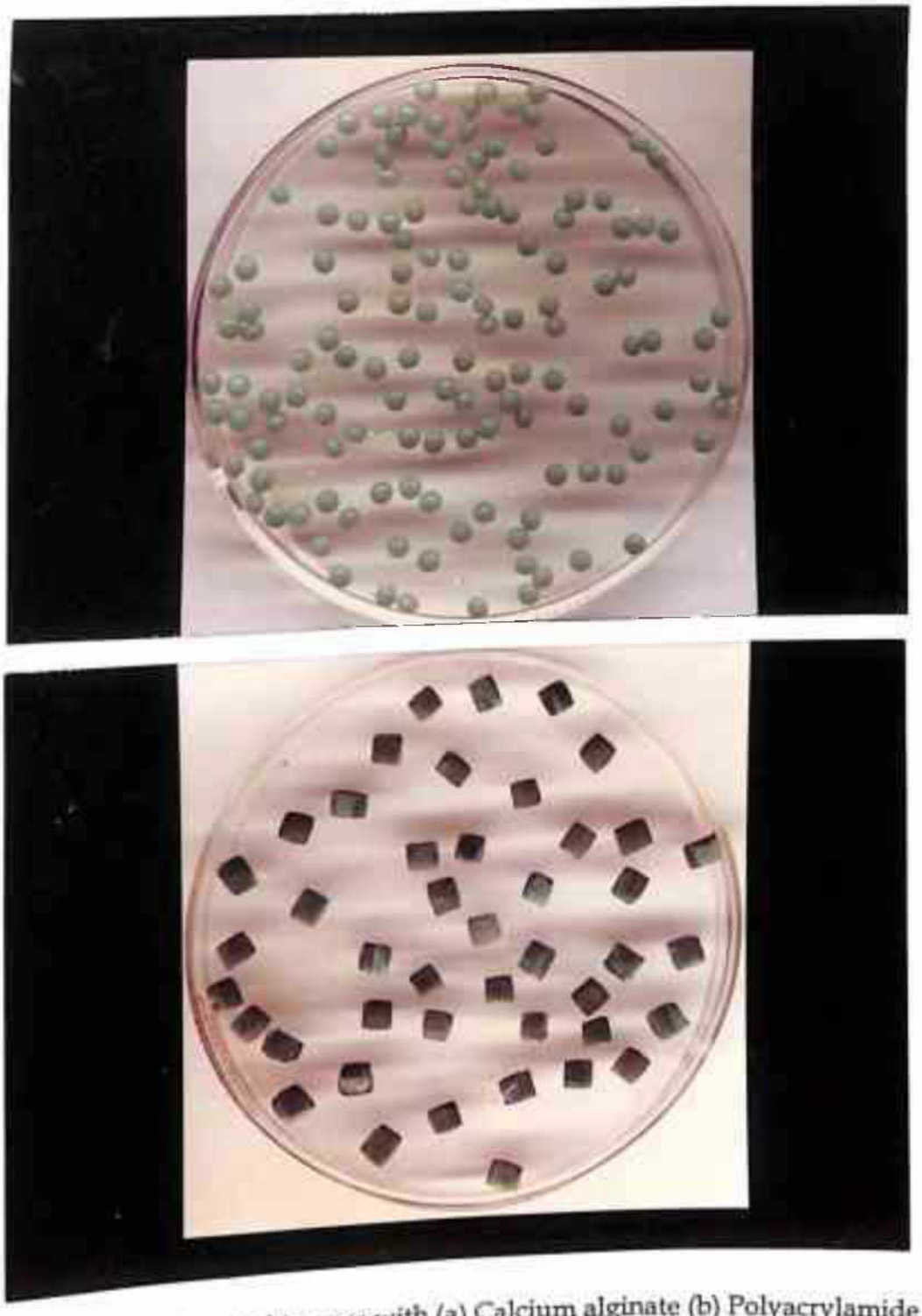

Plate 1. Immobilized *S.platensis* biomass with (a) Calcium alginate (b) Polyacrylamide gel.

13. Characterization of immobilized biomass

13.1. Biomass Loading

To optimize the biomass loading into respective matrix, increasing concentrations of *S.platensis* biomass (normal or lyophilized) was immobilized to achieve various concentrations of cells in the matrix (4%–46% for Ca Alg matrix and 2.0%-69.0% for PAG matrix). Such beads/cubes were used to test the stability and metal uptake capacities.

13.2. Stability of Ca-Alg beads/PAG cubes

The stability of the Ca-Alg beads/PAG cubes containing biomass were tested by incubating them in 100 mL of metal solution (100 mg L^{-1}) for 72 hr on a rotary shaker under culture room conditions. The leakage of biomass from squares was examined by testing 1 mL of ambient metal solution (in which beads/cubes were suspended earlier) either by 2,3,5-triphenyl tetrazolium chloride (TTC) or plating them on 1.8% agar for 48 hr under culture room conditions. The formation of pink colour with addition of TTC or appearance of colonies on the agar plate was considered as the positive indication of cell leakage.

14. Elution of metal

The elution of Co^{2+}, Cu^{2+} and Zn^{2+} by different mineral acids, organic acids, inorganic salts and chelating agent was studied by treating the metal loaded biomass as described for Uranium recovery (Hu and Reeves 1997). The *S.platensis* cells (either in the free form or immobilized form) earlier loaded with metal at 100 mg L^{-1} were washed with deionised distilled water through repeated centrifugation before incubating with eluant solution for 10 min. The cells were then separated from the reaction mixture through

vaccum filtration using 0.45 μm cellulose membrane filter and washed with deionised distilled water. The amount of metal present in this fraction was measured and it was found to be negligible. The metal concentration in the filtrate was measured in the Atomic Absorption Spectrophotometer as described earlier and expressed as percent elution against the metal uptake by the cells. The optimal time required for Co^{2+}, Cu^{2+} and Zn^{2+} elution was determined by drawing the samples at different time intervals (1-10 min) from the reaction mixture followed by its analysis for percent elution.

15. Multiple cycles of sorption and elution

This study was conducted with fixed amount of biomass (50 mg dry weight in immobilized form) was subjected to 100 mg L^{-1} of metal for the desired period after which the biomass bound metal was eluted using 10 mM $CaCl_2$ for Co^{2+} and Zn^{2+}, Ca $(NO_3)_2$ for Cu^{2+} respectively. The loading and elution cycle was repeated in the same manner for a total of 10 cycles without loosing biomass. In the case of column (packed with 2 g of PAG immobilized *S.platensis* biomass; details of the same are mentioned below), a concentration 0.1 mg L^{-1} of Cu^{2+} was pumped. After complete sequestration of the metal ion by the biomass, elution was carried out using 10 mM Ca $(NO_3)_2$. Such cycles of sorption and elution were carried out for total of ten times in order to examine the reusability of columnar reactor packed with PAG immobilized *S.platensis* biomass as mentioned below.

16. Studies with packed bed column reactor

The dimensions of the glass column (Fig.1) used in the present biosorption study were 32 cm in length with an internal diameter of 2.3 cm. The column and its fittings were washed with 10% HNO_3 followed by thorough-rinsing with deionised distilled water before the experiment. The column was loosely packed with 320 PAG squares

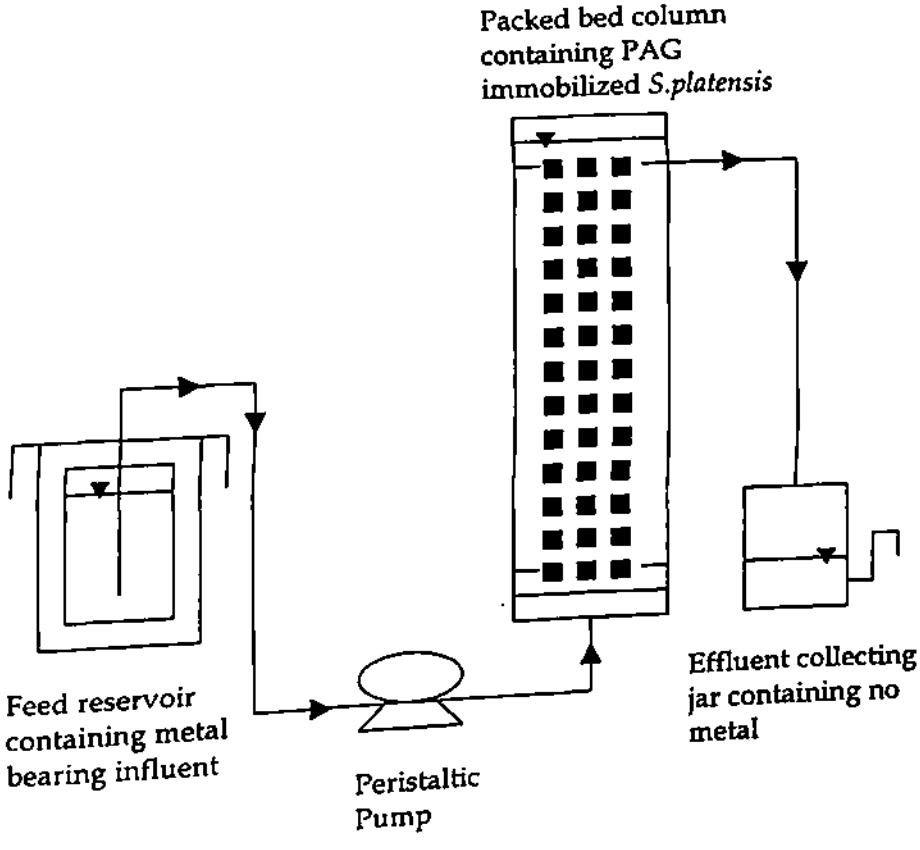

Fig. 1. A schematic representation of up-flow packed bed columnar reactor.

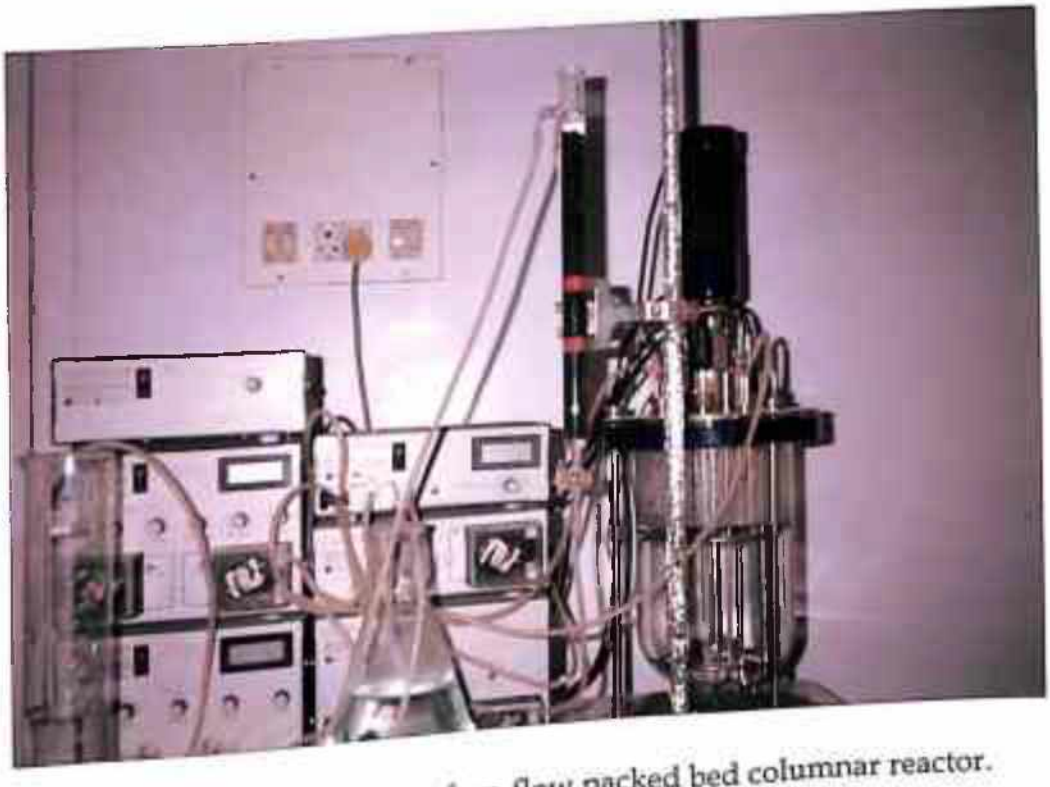

Plate 2. Complete assembly of up-flow packed bed columnar reactor.

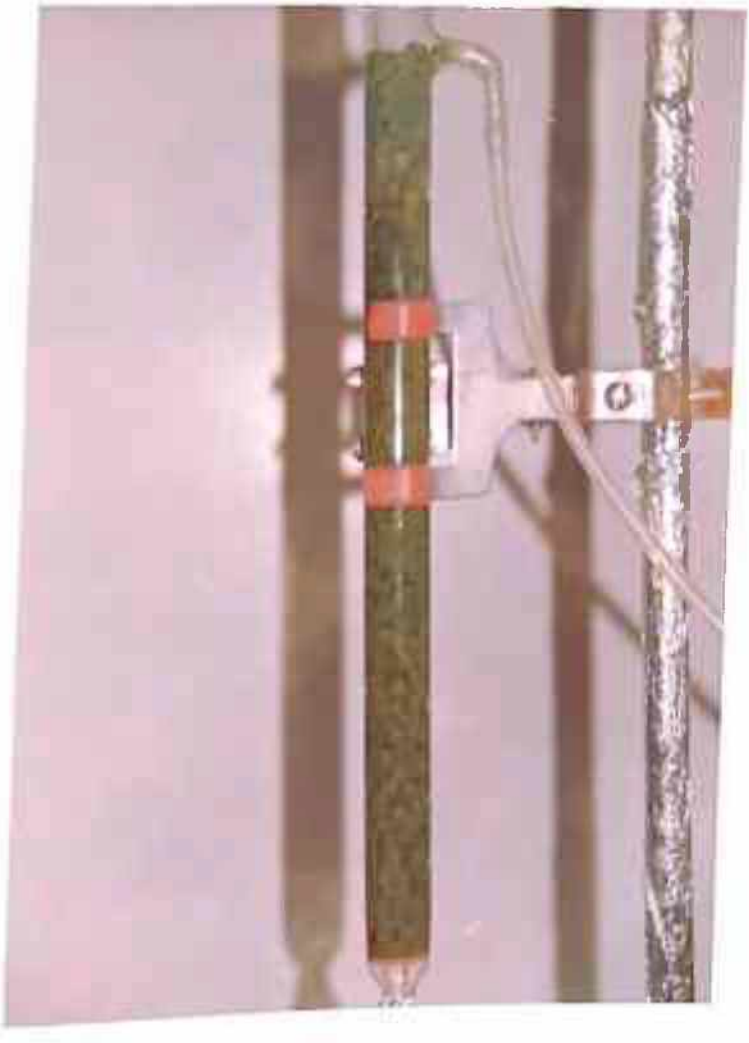

Plate 3. A close-view of columnar reactor packed with PAG immobilized *S.platensis*.

containing approximately 2.0 g of lyophilized *S.platensis* biomass (27.9% w/w). A sufficient quantity of distilled water was pumped through the column prior to run in order to wash the packing material and also to minimize the air pocket formation. Influent solution contained either Cu^{2+} alone or a mixture of Cu^{2+} and Zn^{2+} (in the ratio of 1:22, in view of a typical electro plating industrial wastewater; Schneider and Rubio 1999) were prepared using deionised distilled water (pH 5.90 ± 0.05). The results are expressed in terms of breakthrough curves (obtained by plotting the volume of the influent against the metal concentration found in reactor effluent) and elution curves. The column regeneration was performed by pumping 10 mM Ca $(NO_3)_2$ or $CaCl_2$ for Cu^{2+} and Zn^{2+} respectively. The samples were collected every 1-2 min for analysis using Atomic Absorption Spectrophotometer. The studies involving simulated radionuclide removal of ^{65}Zn and $^{85+89}Sr$ was conducted using 0.1 mg L^{-1} of Zn^{2+} and 2 mg L^{-1} of Sr^{2+}. The values presented are the average of two experiments unless otherwise specified. The complete assembly of the up-flow packed bed columnar reactor is shown in Plate 2 and 3.

17. Studies on localization of accumulated metal

17.1. Study with scanning electron microscope

The *S.platensis* cells exposed to Cu^{2+} (600 mg L^{-1} for 2 hr) were concentrated by centrifugation, washed several times with Na-phosphate buffer (0.1 M, pH 7.4) and fixed by submerging them in 2.5% (w/v) glutaraldehyde-Na phosphate buffer for 2 hr at room temperature. The samples were washed repeatedly with the same buffer, dehydrated in a graded ethanol series (50 to 100%, w/v) and subjected to critical point drying. These were then placed on a carbon coated aluminium stub and gold coated at 0.06-0.08 mbar for 45 seconds followed by viewing under a scanning electron

microscope (*LEO* 435 VP). Sections were examined and compared with metal free control cells as described by Small *et al.* (1999).

17.2. Study with transmission electron microscope

Transmission electron microscopic study was conducted according to the method described by Figueira *et al.* (1999). The *S.platensis* cells exposed to Cu^{2+} (600 mg L^{-1} for 2 hr) were concentrated by centrifugation, washed several times with Na-phosphate buffer (0.1 M, pH 7.4) and fixed by submerging them in 2.5% (w/v) glutaraldehyde-Na phosphate buffer for 2 hr at room temperature. The samples were washed repeatedly with the same buffer and dehydrated in a graded ethanol series (50 to 100%, w/v) and acetone (100%, w/v). Unstained ultrathin sections cut by a Reichart Ultracut E Ultra microtome (Reichart, Germany) with diamond knives and mounted on 100 mesh aluminium grids. Sections were examined and compared with metal free control by loading them in formvar carbon-coated grid in a Philips CM 100 transmission electron microscope at 100 kV.

17.3. Study with IR spectroscope

To obtain a qualitative and preliminary analysis of the main chemical groups present on the cell wall and its contents, an IR analysis in solid phase was performed on lyophilized biomass in a KBr disk (Fourest and Volesky 1996, Pagnanelli *et al.* 2000) using a JASCO IR report- 100 Spectrometer. The IR spectra obtained at 4000-400 cm^{-1} range was used in examining the biomass before and after metal treatment.

18. Statistical analysis

Mean and standard errors have been calculated for the data obtained in different experiments and the variation found to be in the range of 4-5%. The values are the mean of three replicates unless otherwise specified.

Standard error (SE)

The standard error (SE) of the data on specific observation was calculated as,

$SE = SD/\sqrt{n}$

Where, SD = Standard deviation and

n = Number of variants

Correlation coefficient (r^2)

The correlation coefficient (r^2) was calculated for specific data to determine correlation between the two variables ('X' and 'Y') using Microsoft Excel 2000 ®.

Chapter III
RESULTS

1. Selection of suitable strain for metal tolerance and accumulation

1. 1. Determination of toxic concentrations of heavy metals for cyanobacteria

In an effort to examine the toxic concentrations of Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Cs^{2+} and Sr^{2+} for six different cyanobacterial strains, the exponentially grown cells (OD_{560} = 1.8 ≅ 0.99 mg mL^{-1} dry wt.) were added to 50 mL of respective growth medium containing the various concentration of metals in triplicate as mentioned earlier. Among the cyanobacterial strains examined, *Spirulina platensis* was found to tolerate higher amount of metal as it exhibited normal growth at higher concentrations of heavy metals than the other strains examined. *S.platensis* was found to be least sensitive (could tolerate higher metal concentration) towards Mn^{2+}, Fe^{3+}, Zn^{2+}, Cs^{2+} and Sr^{2+}, and was highly sensitive towards Co^{2+}, Ni^{2+}, Cu^{2+} and Cd^{2+} (Table-3). The toxic concentration of these metals varied with the cyanobacterial strain and the type of metal ion used as shown below:

Mn^{2+} *A.fertilisma* = *Aphanothece* sp. = *N.muscorum* = *S.platensis* > *A.cylindrica* = *N.calcicola*

Fe^{3+} *S.platensis* > *N.calcicola* > *A.cylindrica* > *A.fertilisma* = *Aphanothece* sp. = *N.muscorum*

Co^{2+} *A.fertilisma* > *A.cylindrica* = *Aphanothece* sp. = *N.calcicola* = *N.muscorum* = *S.platensis*

Ni^{2+} *A.cylindrica* > *A.fertilisma* = *N.muscorum* > *S.platensis* > *N.calcicola* > *Aphanothece* sp.

Table-3. Toxic concentrations of heavy metal ions for different strains of cyanobacteria.

Strain	*Toxic concentrations (mg L^{-1})*								
	Mn^{2+}	Fe^{3+}	Co^{2+}	Ni^{2+}	Cu^{2+}	Zn^{2+}	Cd^{2+}	Cs^{2+}	Sr^{2+}
A. cylindrica	75.0	15.0	1.0	2.75	0.75	1.25	1.25	800.0	1800.0
Aphanothece sp.	100.0	10.0	1.0	0.75	1.5	1.0	2.5	500.0	1500.0
A.fertilisma	100.0	10.0	2.5	2.5	0.5	1.0	1.0	500.0	1500.0
N.calcicola	75.0	20.0	1.0	1.0	0.75	1.0	7.5	400.0	1800.0
N. muscorum	100.0	10.0	1.0	2.5	0.5	1.0	1.0	400.0	1500.0
S.platensis	100.0	50.0	1.0	2.0	0.5	1.0	5.0	1000.0	1900.0

Cu^{2+} *Aphanothece* sp. > *A.cylindrica* = *N.calcicola* > *A.fertilisma* = *N.muscorum* = *S.platensis*

Zn^{2+} *A.cylindrica* > *A.fertilisma* = *Aphanothece* sp. = *N.calcicola* = *N.muscorum* = *S.platensis*

Cd^{2+} *N.calcicola* > *S.platensis* > *Aphanothece* sp. > *A.cylindrica* > *A.fertilisma* = *N.muscorum*

Cs^{+} *S. platensis* > *A.cylindrica* > *A.fertilisma* = *Aphanothece* sp. > *N.calcicola* = *N. muscorum*

Sr^{2+} *S.platensis* > *A.cylindrica* = *N.calcicola* > *A.fertilisma* = *Aphanothece* sp. = *N.muscorum*

1. 2. Metal accumulation studies

The cyanobacterial strains were exposed to Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} and Sr^{2} in order to choose the suitable candidate for metal biosorption process. The exponentially grown cells of different strains of cyanobacteria (ca. 50 mg) were subjected to varying metal concentration (50-800 mg L^{-1}). The samples were collected after 2 hr of incubation and assayed for metal concentration using an Atomic Absorption Spectrophotometer and the results are expressed in terms of maximum metal uptake. Among the tested strains, *S.platensis* exhibited maximum metal accumulation of Cu^{2+} (272 mg g^{-1} biomass) followed by Zn^{2+} (252 mg g^{-1}), Co^{2+} (181 mg g^{-1}), Cd^{2+} (95.6 mg g^{-1}), Ni^{2+} (77.2 mg g^{-1}) and Sr^{2+} (39.4 mg g^{-1}), within 2 hr of metal exposure (Table-4). The other strains exhibited significantly lower accumulation of these metal, closest being *Nostoc calcicola* with 89.3 mg g^{-1} for Cu^{2+}, 112.3 mg g^{-1} for Zn^{2+}, 121.3 mg g^{-1} for Co^{2+}, 90.1 mg g^{-1} for Cd^{2+}, 71.6 mg g^{-1} for Ni^{2+}, 26.2 mg g^{-1} for Sr^{2+}. Thus, *S.platensis* with higher metal accumulation

Table-4. Metal accumulation by different strains of cyanobacteria.

Strain	*Metal accumulation (mg g^{-1})*					
	Co^{2+}	Ni^{2+}	Cu^{2+}	Zn^{2+}	Cd^{2+}	Sr^{2+}
A.cylindrica	46.3	59.3	79.6	85.1	57.2	20.3
Aphanothece sp.	27.6	17.2	54.1	29.3	9.3	5.3
A.fertilisma	76.3	42.1	22.3	12.6	29.4	2.3
N.calcicola	121.3	71.6	89.3	112.3	90.1	26.2
N. muscorum	110.3	66.2	56.3	42.1	32.3	9.6
S.platensis	181.0	77.2	272.0	252.1	95.6	39.4

and its low metal sensitivity was selected as a suitable strain for detailed metal biosorption/bioaccumulation studies.

1. 3. General growth characteristics of *S.platensis*

The growth pattern of *S.platensis* was monitored in medium recommended by Ogawa and Terui (1970). The growth rate with the initial inoculum of 50 μg protein mL^{-1} showed a negligible lag phase (1 d) followed by a sharp exponential phase extending for 7 d and a slow late log phase (till 9d) corresponding to a final cell yield of ca. 4.06 mg protein mL^{-1}. The over all pattern showed a generation time (g) of 1.2 d with specific growth rate constant (k) of 0.83 (Fig. 2).

2. Uptake of Co^{2+}, Cu^{2+} and Zn^{2+} by normal *Spirulina platensis* biomass

The uptake rate of Co^{2+}, Cu^{2+} and Zn^{2+} was determined considering various parameters, viz., pH, biomass density, metal concentration, and its kinetics, light, temperature and presence of co-ions.

2. 1. Role of pH on metal uptake

The role of pH in metal (Co^{2+}, Cu^{2+} and Zn^{2+}) precipitation was estimated indirectly by measuring the metal ion present in the final reaction mixture at different pH (3.0 – 9.0). pH of the solution was adjusted to the desired value by adding the required amount of either 0.1N HCl or 0.1N NaOH at the beginning of the experiment. The metal ion concentration in general remained unchanged in pH less than or equal to 6.0 followed by metal precipitation. The Co^{2+} concentration remained unaltered till pH 6.5 followed by 5.0%–7.0% precipitation at pH 7.0. Precipitation of the Co^{2+} increased to 10.0% and 20.0% when pH was raised to 8.0 and 9.0 respectively. On the other hand, $Cu^{2+}$and Zn^{2+} showed

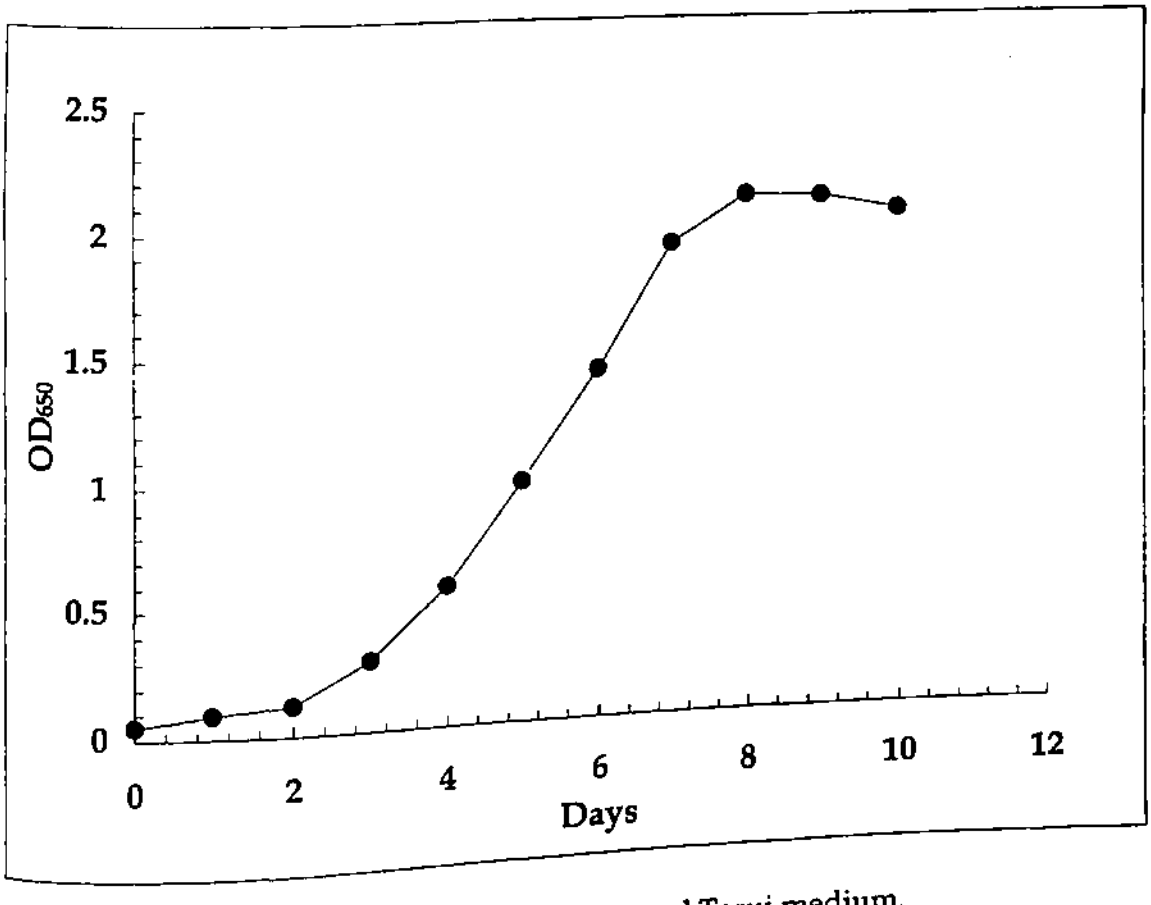

Fig.2. Growth pattern of *S.platensis* in Ogawa and Terui medium.

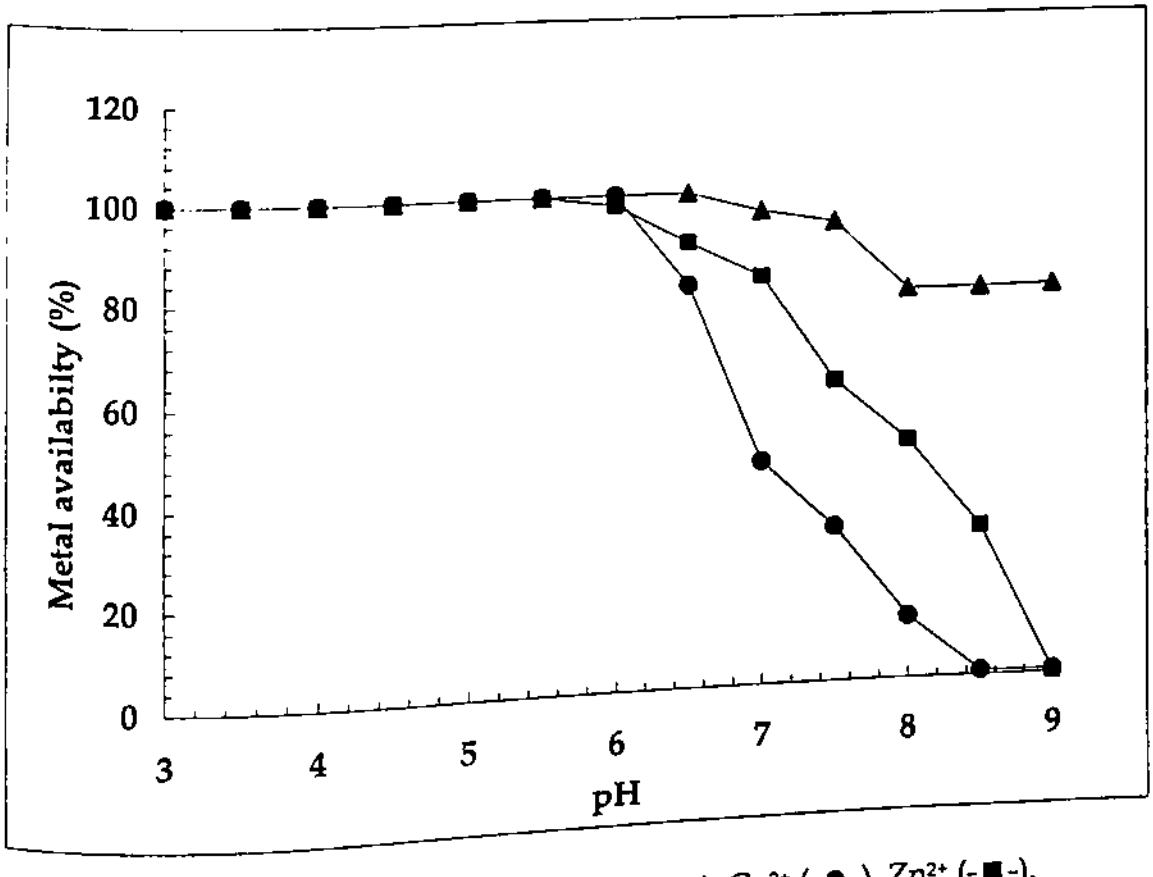

Fig. 3. Effect of pH on metal availability; Co^{2+} (-▲-), Cu^{2+} (-●-), Zn^{2+} (-■-).

20.0% precipitation even at pH 7.0, which further increased to 90.0% in case of Cu^{2+} and 65.0% in case of Zn^{2+} at pH 8.0. The Cu^{2+} and Zn^{2+} remained undetectable at pH beyond 9.0 due to complete precipitation. However, only 30% of Co^{2+} precipitation was observed at pH 9.0 (Fig. 3). The metal uptake pattern by the *S.platensis* biomass showed a continuous increase in uptake with increasing pH in the acidic range reaching to a maximum value of 29.1 mg Co^{2+} g^{-1}, 78.0 mg Cu^{2+} g^{-1} and 38.7 mg Zn^{2+} g^{-1} biomass at pH 6.0 (Fig. 4). Observation above pH 6.5 was not conducted because of possible metal precipitation.

2. 2. Effect of biomass density

Since the ligands concentration is directly proportional to the biomass surface area, it was imperative to study the effect of biomass density on metal uptake. The uptake of Co^{2+}, Cu^{2+} or Zn^{2+} by normal cells of *S. platensis* was increased with increase in biomass density (0.5 - 5.0 mg mL^{-1}). Though the total amount of metal uptake increased with increasing biomass concentration there was a decline in the metal uptake values expressed in mg of metal taken by unit weight of biomass. The uptake of Co^{2+} ion decreased by 83.0 % with increasing the biomass density from 0.5 mg mL^{1} to 5.0 mg mL^{-1}. The Cu^{2+} and Zn^{2+} also followed a similar pattern (Fig. 5).

2. 3. Effect of metal concentration

The Co^{2+}, Cu^{2+} or Zn^{2+} uptake was investigated at varying metal concentrations (50-800 mg L^{-1}) by the *S.platensis* biomass within 2 hr of metal exposure. In all the cases examined, metal uptake was found to be dependent on external metal concentration. The Co^{2+} uptake by normal cells of *S.platensis*, a ca. 31 mg Co^{2+} g^{-1} of biomass was noticed at lowest external concentration tested (50 mg L^{-1}). A doubling of external Co^{2+} concentration to 100 mg L^{-1} exhibited an uptake of ca. 83 mg Co^{2+} g^{-1} of biomass. The uptake of Co^{2+} increased

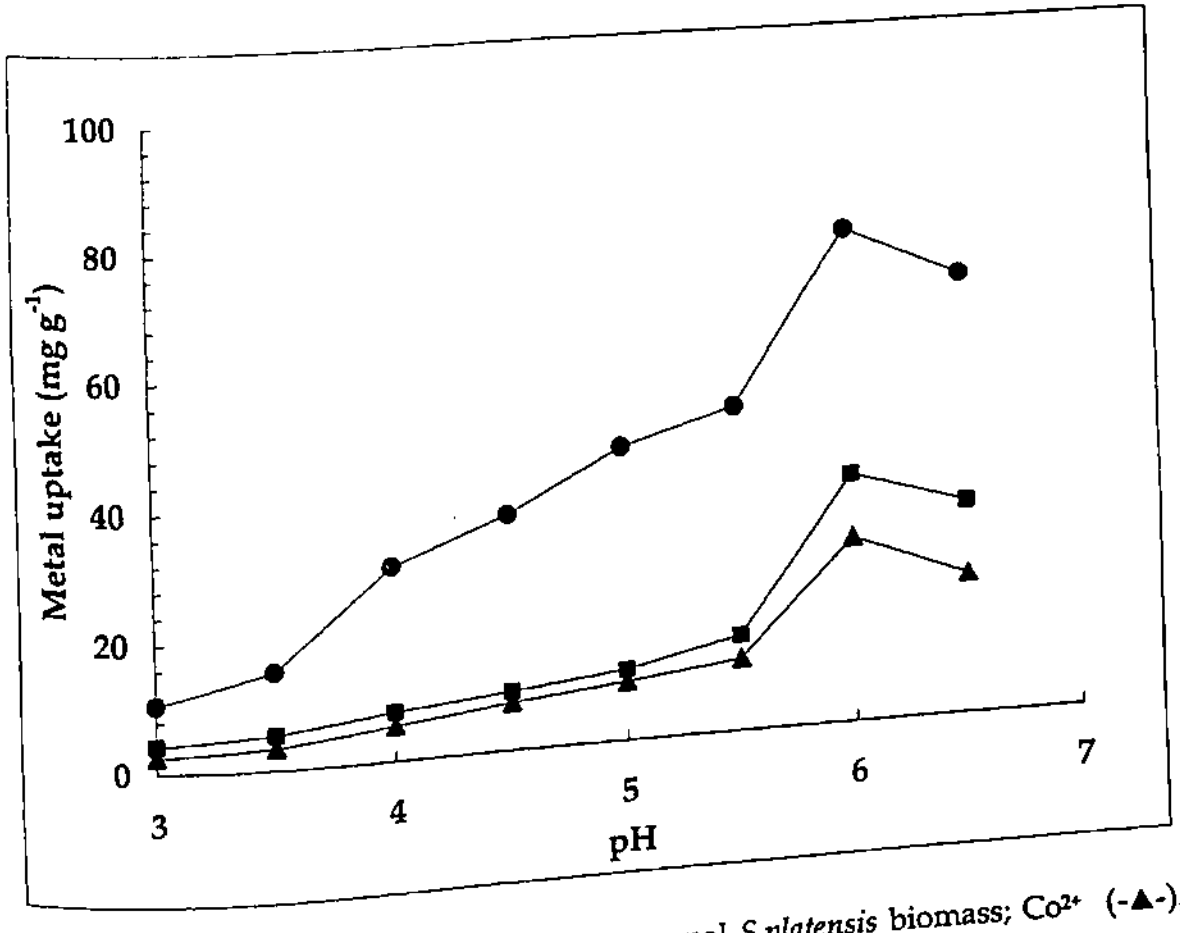

Fig. 4. Effect of pH on metal uptake by normal *S.platensis* biomass; Co^{2+} (-▲-), Cu^{2+} (-●-), Zn^{2+} (-■-).

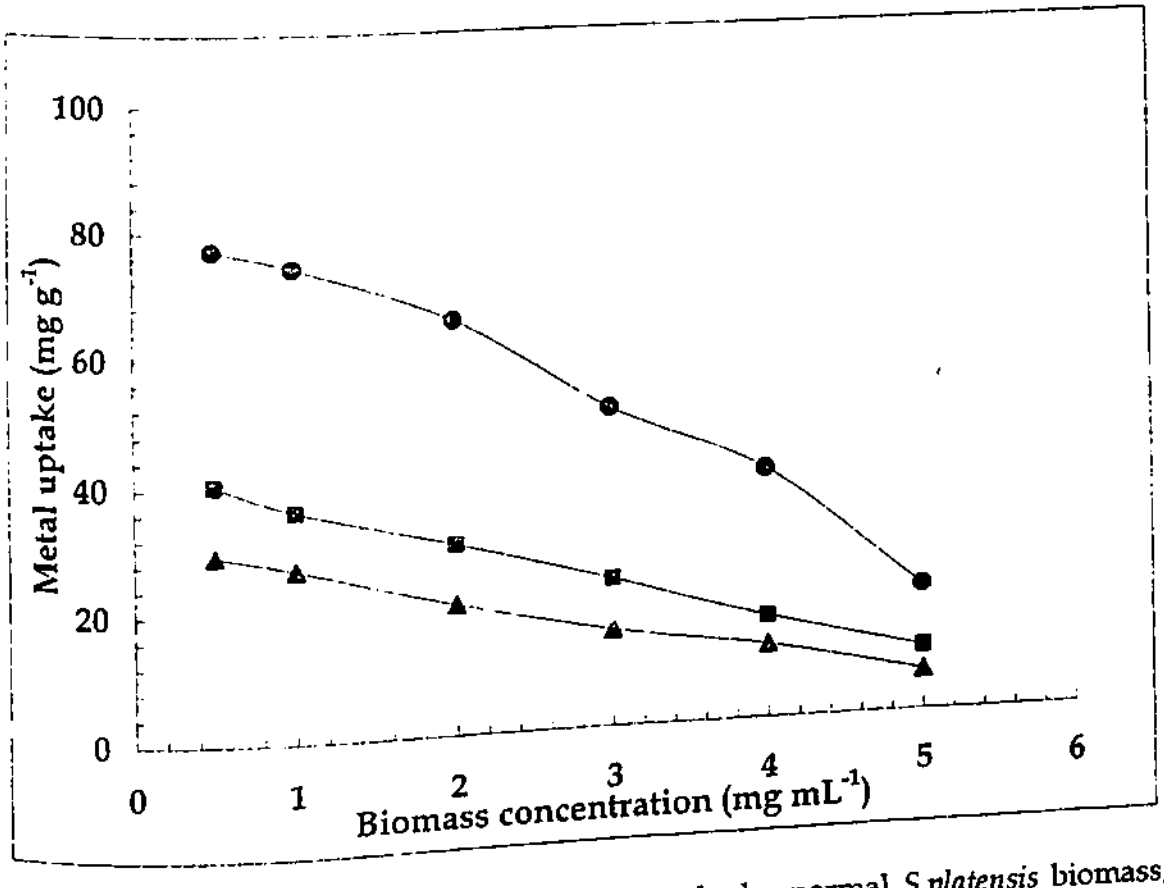

Fig. 5. Effect of biomass density on metal uptake by normal *S.platensis* biomass; Co^{2+} (-▲-), Cu^{2+} (-●-), Zn^{2+} (-■-).

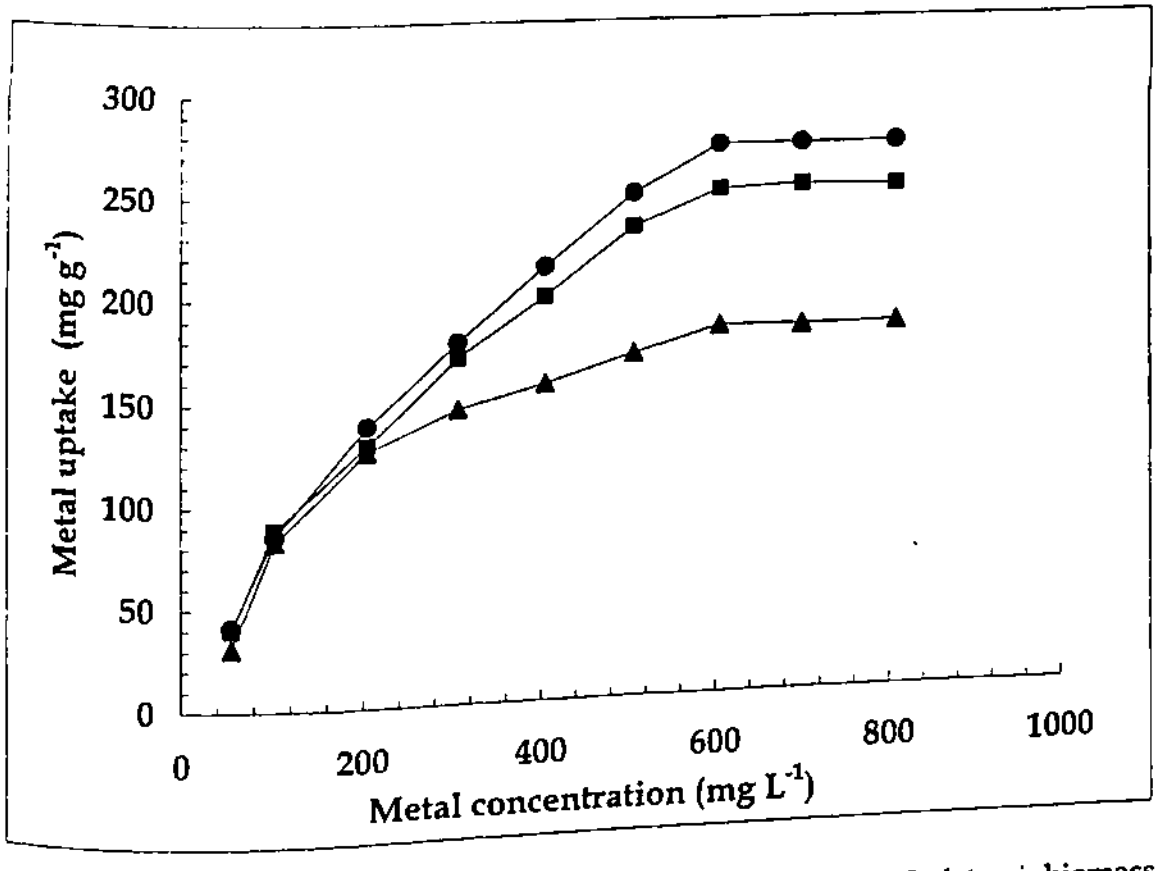

Fig. 6. Effect of metal concentration on metal uptake by normal *S.platensis* biomass; Co^{2+} (-▲-), Cu^{2+} (-●-), Zn^{2+} (-■-).

proportionately with increasing concentration attaining a maximum value of 181 mg Co^{2+} g^{-1} at 600 mg L^{-1} concentration of Co^{2+}. However, further increment in metal concentration did not result in further uptake indicating biomass saturation (Fig. 6). Thus ca. 18% of Co^{2+} accumulation was attained by the biomass on dry weight basis. The Cu^{2+} uptake on the other hand showed a ca. 41 mg Cu^{2+} g^{-1} of biomass at 50 mg Cu^{2+} L^{-1}. A doubling of external Cu^{2+} concentration to ca. 100 mg L^{-1} exhibited an uptake of 85 mg Cu^{2+} g^{-1} of biomass. The saturation in Cu^{2+} uptake (272.0 mg g^{-1} of biomass) was obtained at 600 mg Cu^{2+} L^{-1} concentration showing a total of 27% (w/w) accumulation. The Zn^{2+} uptake also followed the similar pattern with maximum of 25% accumulation (Fig. 6).

2. 4. Kinetics of metal uptake

In order to study the time-dependent metal bioaccumulation, *S.platensis* biomass was subjected to 100 mg L^{-1} metal solutions (Co^{+2}, Cu^{2+} or Zn^{2+}) separately. The metal uptake in terms of percent fraction of total uptake within 2 hr is represented as a function of contact time in Fig. 7. The metal bioaccumulation by normal biomass was found to be bi-phasic phenomenon involving an initial rapid phase followed by slow secondary phase. The process was very rapid in first minute of contact (data not shown) accounting for about 64.0% for Co^{2+} - 65.0% for Cu^{2+} - and 63.0% for Zn^{2+} - of the total uptake. Secondary residual uptake started after 1-2 min of contact and continued for 2 hr followed by a stationary phase indicating equilibrium. No further increment in metal uptake was found even after 24 hr of contact time (Fig. 7).

2. 5. Effect of light

In order to study the effect of photosynthetic light, the uptake mixture containing *S.platensis* biomass and 100 mg L^{-1} of Co^{2+}, Cu^{2+} or Zn^{2+} solution was incubated in either dark as well as culture room conditions. The uptake was studied after 2 hr of metal

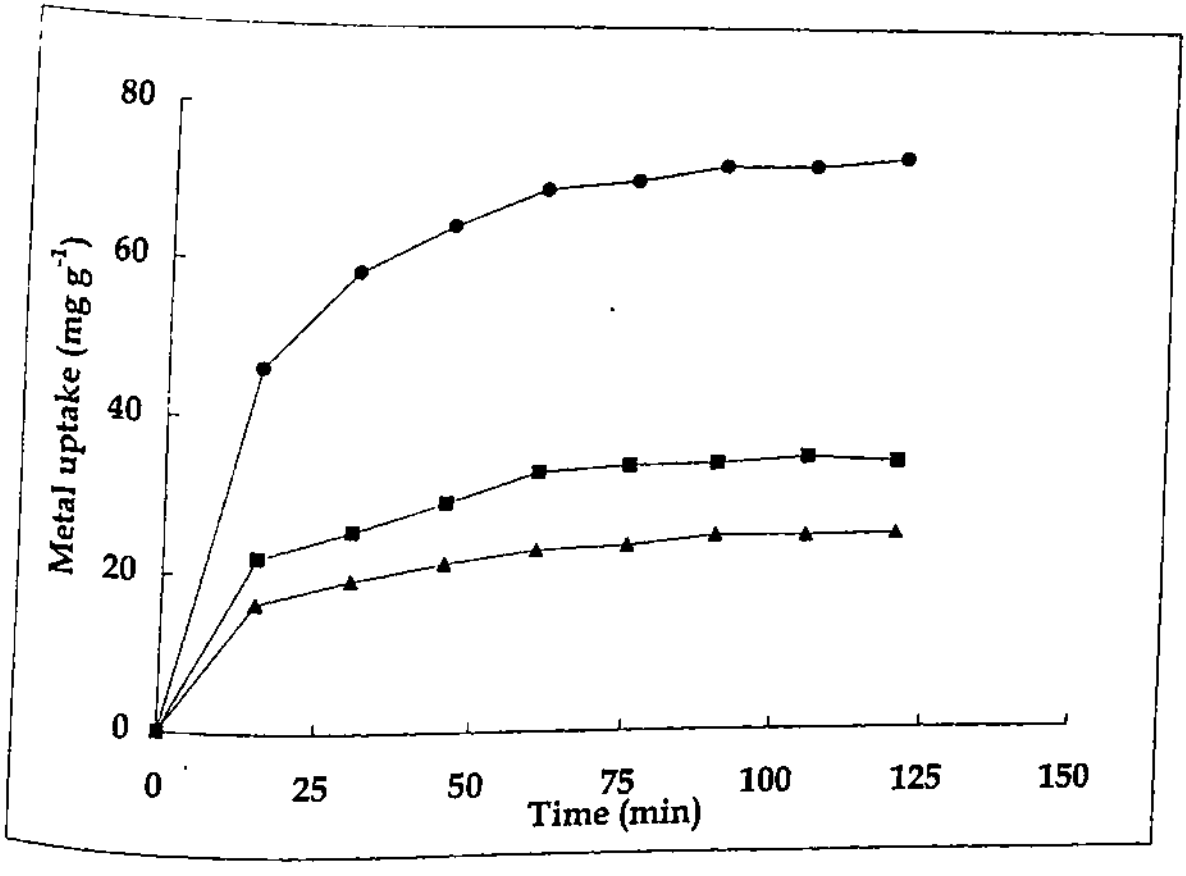

Fig. 7. Kinetics involved in metal uptake by normal *S.platensis* biomass; Co^{2+} (-▲-), Cu^{2+} (-●-), Zn^{2+} (-■-).

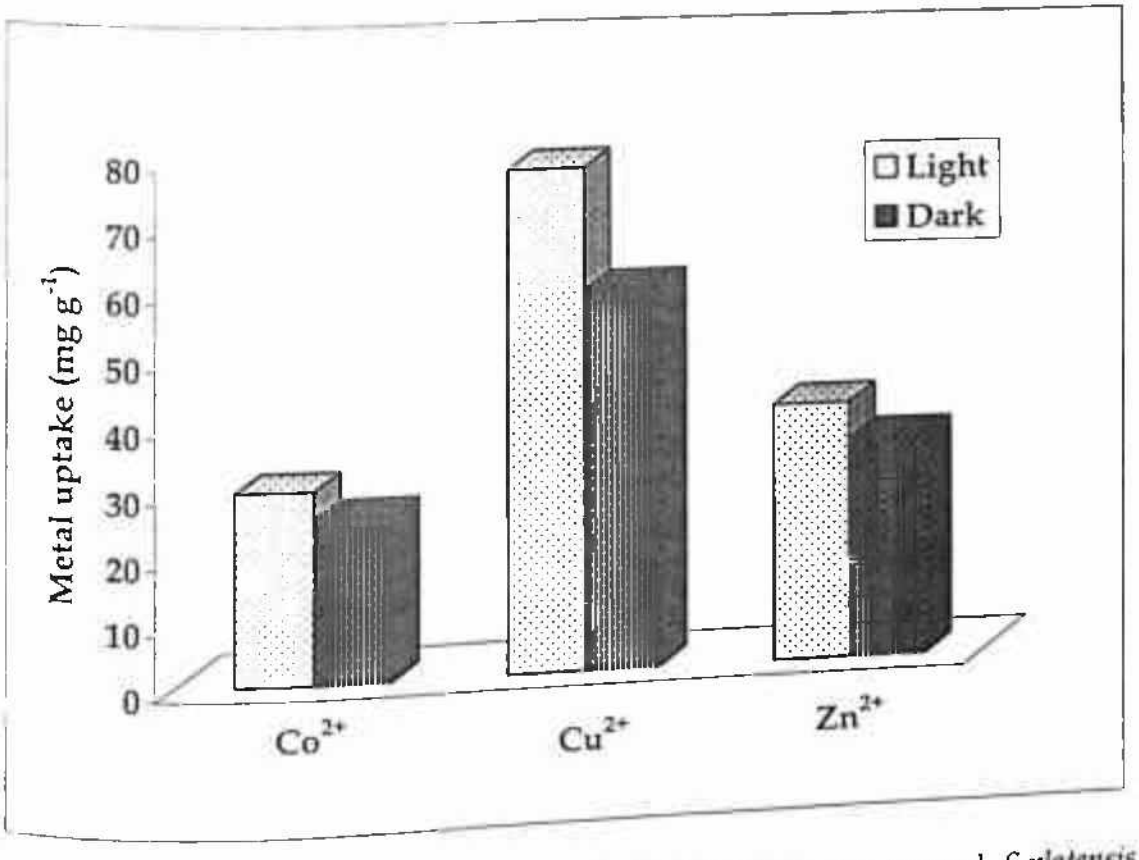

Fig. 8. Metal uptake under light and dark conditions by normal *S.platensis* biomass.

exposure. The metal accumulation was found to decrease under dark conditions as it showed ca. 24% reduction in Cu^{2+} uptake, followed by 14% Co^{2+} and 13% Zn^{2+} uptake (Fig. 8).

2. 6. Effect of temperature

The accumulation of Co^{2+}, Cu^{2+} or Zn^{2+} by normal *S.platensis* biomass was studied at various temperature (10°C-50°C) and results are presented in Fig. 9. No significant difference in metal accumulation was evident in any of the tested metal in the temperature range of 10°C - 30°C. However ca. 10% reduction in metal uptake was observed for all the three metals at 40°C followed by a further reduction of 15% when temperature increased to 50°C.

2. 7. Effect of co-ions

In nature the target metal is usually found in combination with other metal ions, which might influence the microbial interaction with the target metal. In order to examine the effect of other co-ions on the accumulation of target metal ions by the normal *S.platensis* biomass, a series of experiments were designed in bi metallic conditions with their respective controls. The percent reduction in metal uptake by normal *S.platensis* biomass in presence of other cations at equimolar concentration against control is shown in Table-5. The presence of Ni^{2+} exhibited inhibitory effect on uptake of Co^{2+}, Cu^{2+} and Zn^{2+} by normal *S.platensis* biomass contributing to ca. 49%, 3% and 6% reduction in uptake respectively. The Cd^{2+} was found to inhibit the uptake of Co^{2+}, Cu^{2+} and Zn^{2+} to the tune of 11%, 4% and 4% respectively. The effect of Co^{2+}, Cu^{2+} and Zn^{2+} on the cross -uptake was also studied by mixing the target metal with the interacting ion in equimolar concentrations. Cu^{2+} had maximum inhibitory effect on both Co^{2+} and Zn^{2+} uptake as it reduced Co^{2+} and Zn^{2+} uptake to the extent of ca. 55% and 16% respectively. Conversely,

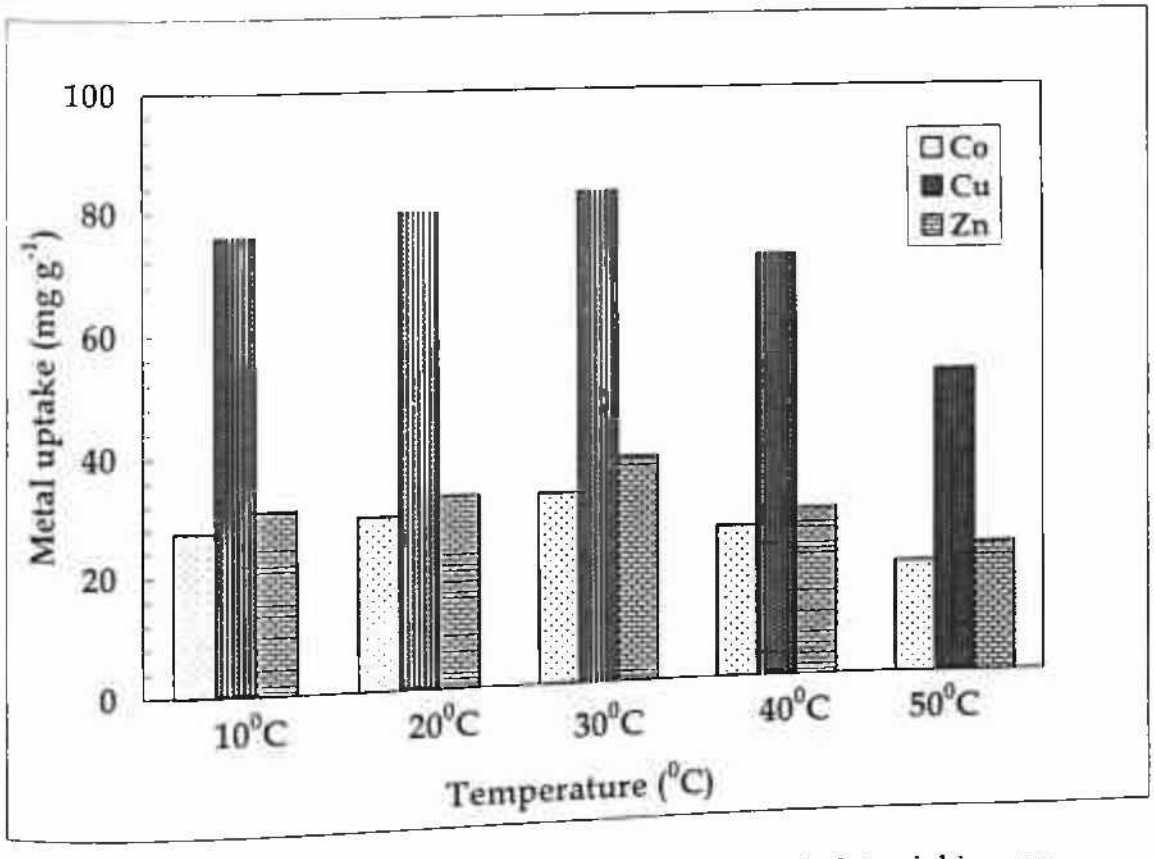

Fig. 9. Effect of temperature on metal uptake by normal *S.platensis* biomass.

Table-5. Effect of co-ions on uptake of Co^{2+}, Cu^{2+} and Zn^{2+} by normal biomass of *S.platensis* in bi-metallic solutions.

Additional cations	*Reduction in metal uptake (%)*		
	Co^{2+}	Cu^{2+}	Zn^{2+}
Control	0.0	0.0	0.0
Co^{2+}	--	1.9	9.9
Ni^{2+}	48.8	2.8	6.3
Cu^{2+}	54.4	--	16.1
Zn^{2+}	43.7	8.7	--
Cd^{2+}	10.8	3.8	3.8

the presence of Co^{2+} reduced the uptake of Cu^{2+} and Zn^{2+} by ca. 2% and 10% respectively. Similarly, the presence of Zn^{2+} was also found to be inhibitory for Co^{2+} (44%) and Cu^{2+} (9%) uptake by normal biomass of *S.platensis*.

2. 8. Studies on cellular localization of accumulated metal

In order to study the localization of accumulated metal by the *S.platensis*, the Cu^{2+} loaded biomass was examined using Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM). The cells were exposed to saturating Cu^{2+} concentration (600 mg L^{-1}) for 2 hr and observed under microscope. The metal treated cells were compared with untreated control cells using the above-mentioned techniques. The Scanning electron micrograph of Cu^{2+} treated cells (Plate 4b) exhibited small filament like out growths in cell wall in response to metal exposure as compared to the smooth surface of cell wall of control cells (Plate 4a). The transmission electron micrograph exhibited that the control cells has few small electron dense bodies in the homogeneous cytoplasm surrounded by the thinner cell envelope (Plate 5a), whereas the metal treated cells showed a distinct, electron-opaque, ring like area at or near the cells periphery indicating the accumulation of Cu^{2+} (Plate 5b).

The functional groups involved in Cu^{2+} accumulation by the *S.platensis* was elucidated using Infra Red (IR) Spectra obtained at 4000-600 cm^{-1} range (Fig. 10). The IR spectrum showed several peaks reflecting complex nature of the *S.platensis* biomass. The native biomass, comprising different functional groups which regulate the possible cell-cation interactions like H-bonding, complexation, etc. selectively gives following information on accumulation of Cu^{2+}. The native biomass exhibited characteristic absorption at 3500-3000 cm^{-1} and 1200-900 cm^{-1}. The first peak lied in a spectrum region occupied by a strong bond (3600-3000 cm^{-1}). The sharp peaks at 3000-2800 cm^{-1}, 1650 cm^{-1}, 1600 cm^{-1}, 1550 and

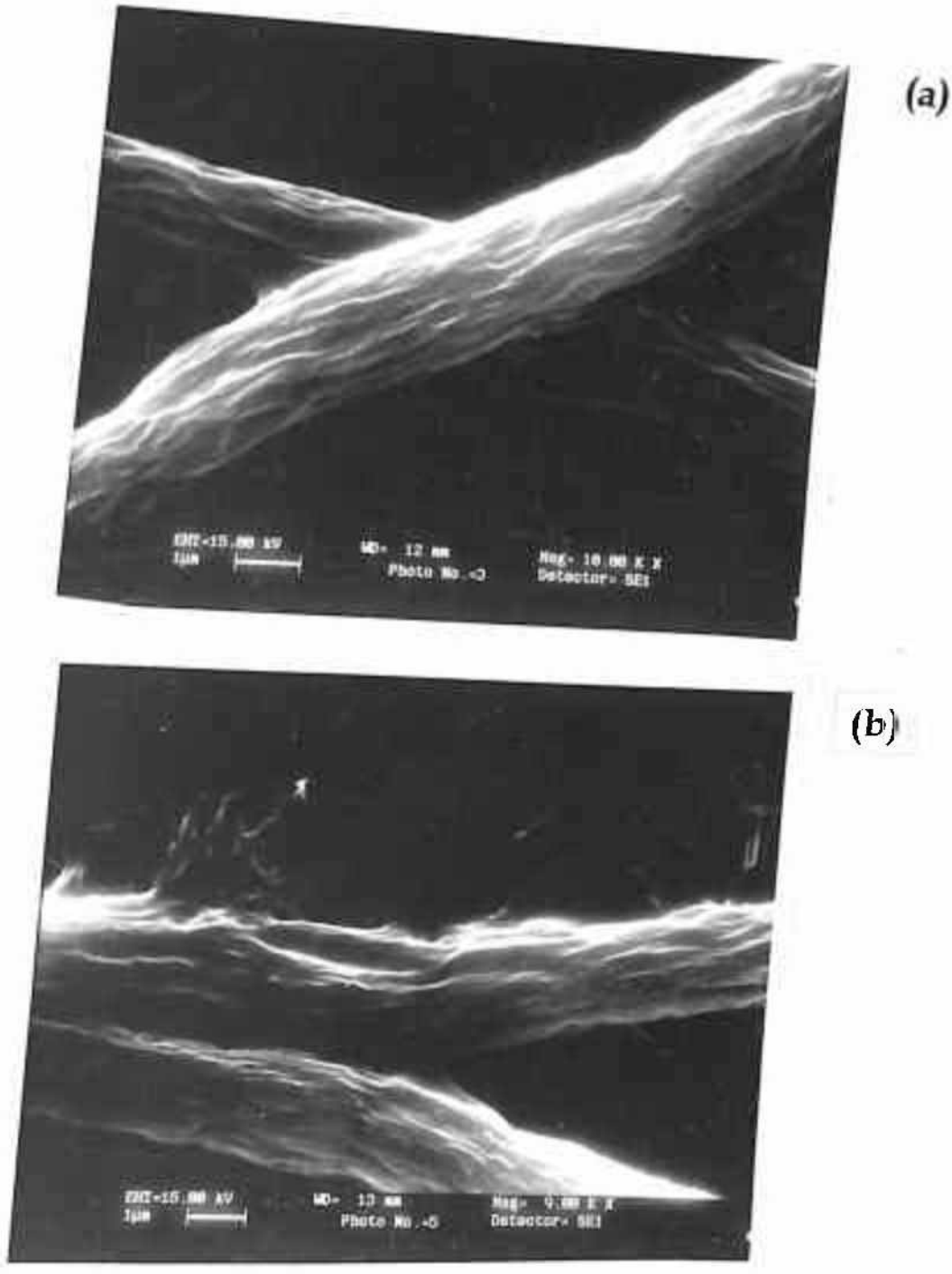

Plate 4. Scanning electron micrographs of *S. platensis* (a) Metal untreated control cell and (b) Cell after 2hr of Cu^{2+} (600 mg L^{-1}) treatment; Magnification x 10 000.

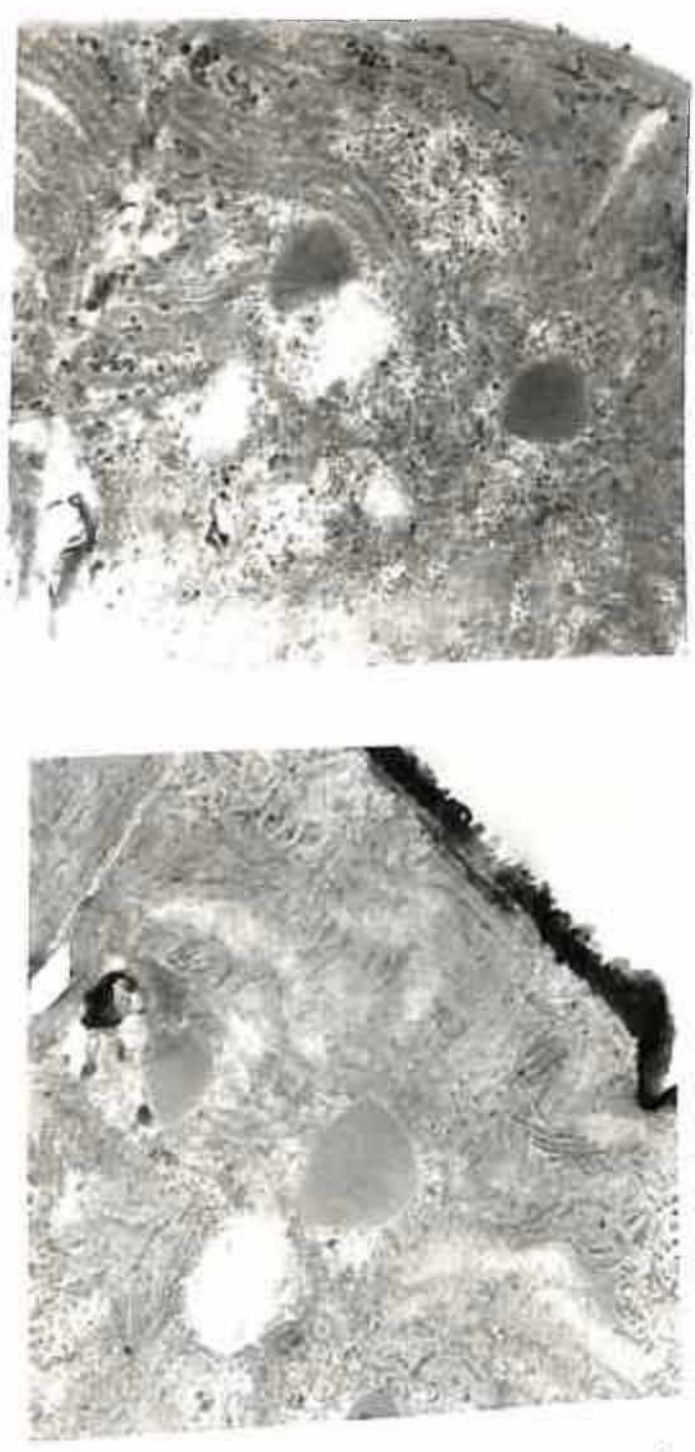

Plate 5. Transmission electron micrographs of *S. platensis* (a) Metal untreated control cell and (b) Cell after 2hr of Cu^{2+} (600 mg L^{-1}) treatment; Magnification x 10 000.

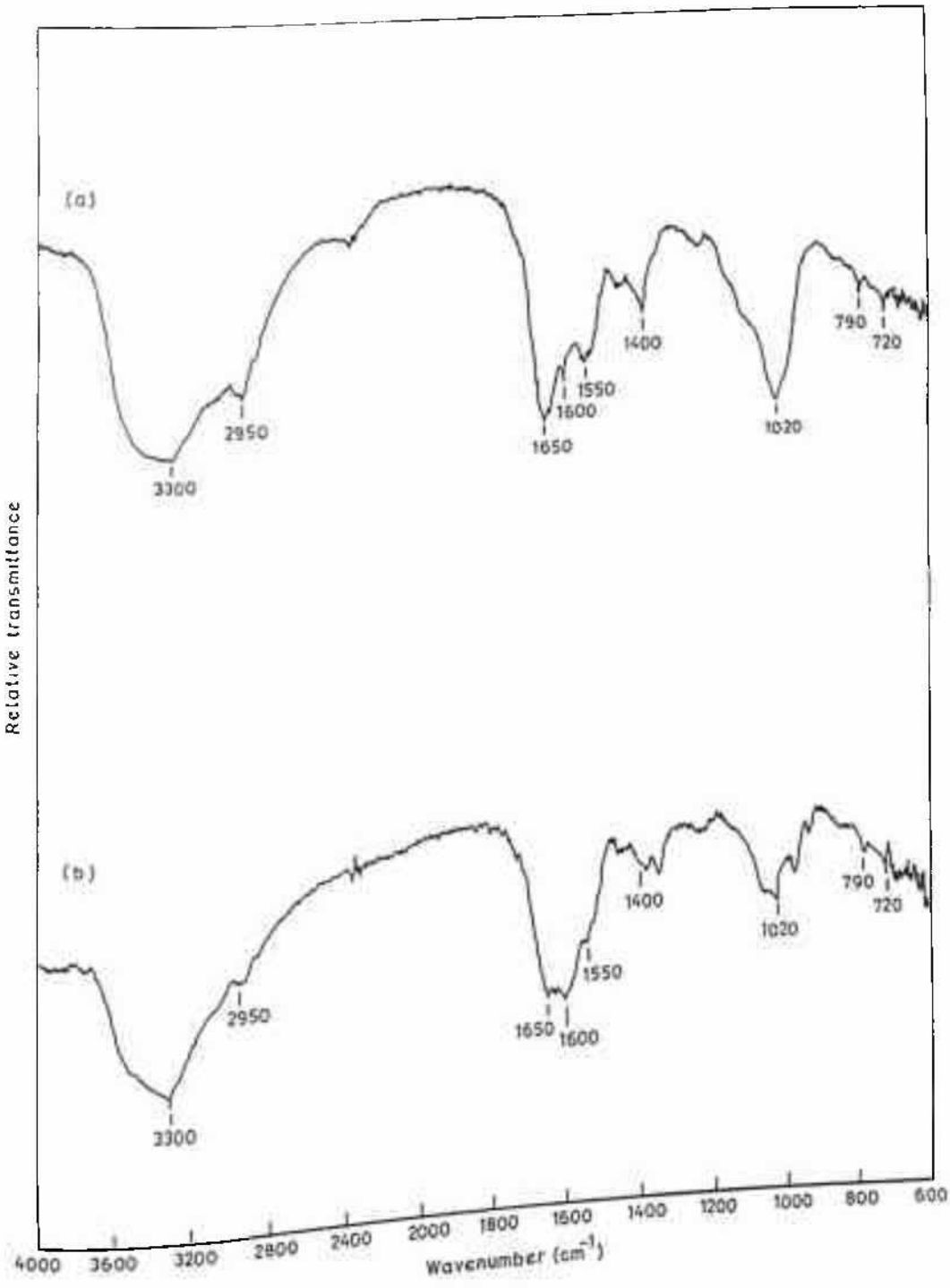

Fig. 10. Infra-red spectra of *S.platensis* biomass (a) Metal untreated biomass and (b) Biomass after 2hr of Cu^{2+} (600 mg L^{-1}) treatment.

1400 cm^{-1} were also seen in the spectrum. The IR spectrum of metal treated cells indicated no such shifts or change in any of the characteristic absorbance bands exhibited by the native biomass. The only discernible changes observed were in the length, width and intensities of the peaks. Especially, there was a 40-45%reduction in peak lengths that belong to the 1650 and 1020 cm^{-1} bands. A major change in sharpness and intensity of the peak found in the 1650 cm^{-1} band. There was minor change in peak intensity of band related to 1550 cm^{-1}.

3. Uptake of Co^{2+}, Cu^{2+} and $Zn^{2+}$by lyophilized and oven-dried *Spirulina platensis* biomass

3. 1. Effect of pH

As shown in the previous studies, the pH of ambient medium influenced the availability of metal in the solution, the sorption of Co^{2+}, Cu^{2+} and Zn^{2+} by lyophilized (freeze-dried) as well as oven-dried *S. platensis* biomass was investigated at varying pH (3.0 – 6.5). The metal sorption pattern by the both forms of *S.platensis* biomass showed a regular increase in the metal uptake with increasing pH in the acidic range attaining a maximum uptake value of 27.2 mg Co^{2+} g^{-1}, 72.3 mg Cu^{2+} g^{-1} and 41.3 mg Zn^{2+} g^{-1} by lyophilized biomass at pH 6.0. The oven-dried biomass, on the other hand showed a significantly lower uptake of Co^{2+} (12.7 mg g^{-1}), Cu^{2+} (31.0 mg g^{-1}) and Zn^{2+} (15.5 mg g^{-1}) as compared to the lyophilized biomass (Fig. 11) at pH 6.0. Observations above pH 6.5 were not possible because of metal precipitation.

3. 2. Effect of biomass density

In order to study the effect of available surface area of the biomass, varying density of lyophilized as well as oven-dried *S.platensis* (0.5 mg mL^{-1} – 5.0 mg mL^{-1}) was used in the sorption mixture. Although the total metal sorption increased with increasing biomass

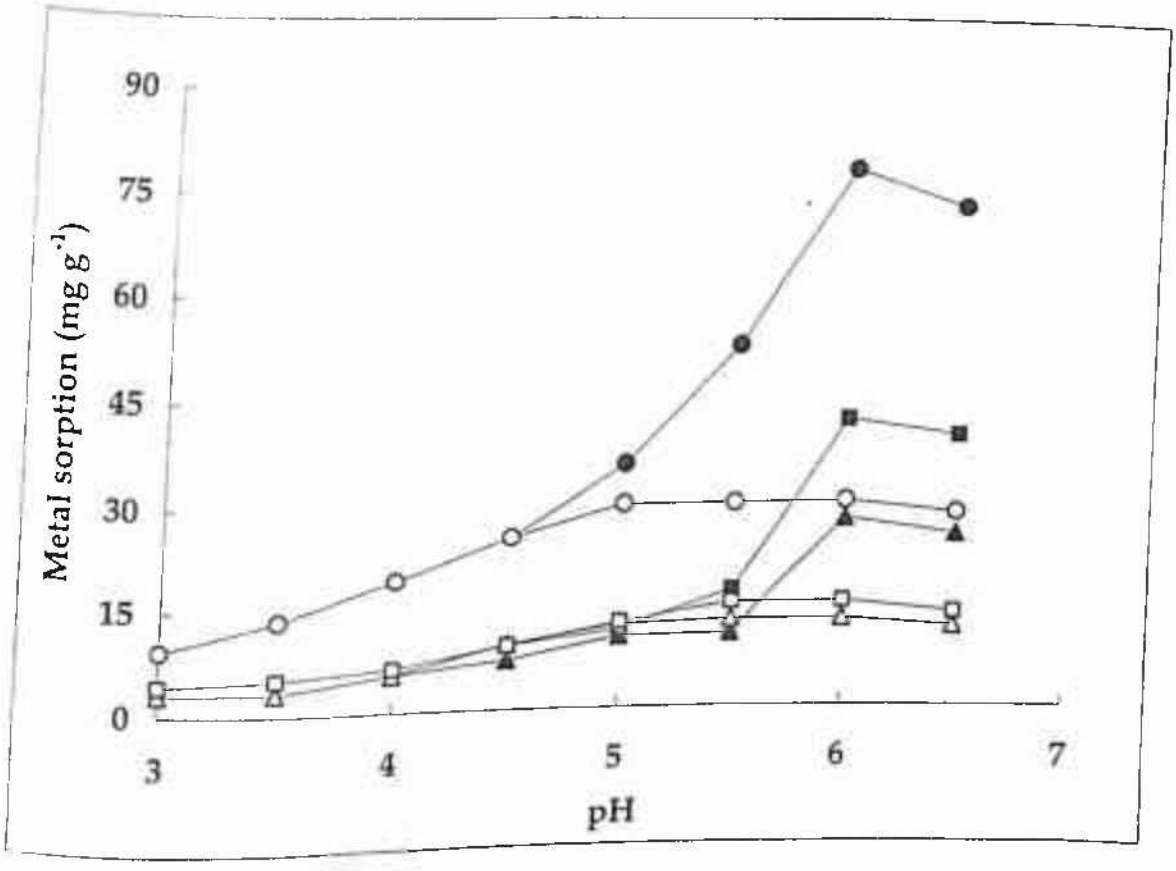

Fig. 11. Effect of pH on metal sorption by lyophilized and oven-dried *S.platensis* biomass; Co^{2+} (-▲-), Cu^{2+} (-●-), Zn^{2+} (-■-) sorption by lyophilized biomass; Co^{2+}(-△-), Cu^{2+} (-○-), Zn^{2+} (-□-) sorption by oven-dried biomass.

density, but there was a net decrease in metal sorption per unit weight of biomass as was observed in normal biomass. A ten-fold increase in lyophilized biomass density from 0.5 mg L^{-1} to 5.0 mg L^{-1} exhibited a marked reduction in Co^{2+} (ca. 60%), Cu^{2+} (ca. 69%) and Zn^{2+} (ca. 75%) sorption. Similarly a ten-fold increase in oven-dried biomass density in the reaction mixture showed a reduction of ca. 80%, 71% and 65% for Co^{+2}, Cu^{2+} and Zn^{+2} sorption respectively (Fig. 12).

3. 3. Equilibrium metal sorption

The sorption performance of Co^{2+}, Cu^{2+} and Zn^{2+} by lyophilized or oven-dried biomass of *S.platensis* was ascertained by the biosorption equilibrium isotherms at varying metal concentrations of 50-800 mg L^{-1}. The data is presented in terms of its metal loading as a function of residual metal concentrations (equilibrium concentration, Ce). At the lowest Co^{2+} equilibrium concentration (42.0 mg L^{-1}), lyophilized biomass showed 12.6 mg Co^{2+} g^{-1} sorption whereas oven-dried biomass showed only 5.1 mg Co^{2+} g^{-1} of biomass. However, Co^{2+} sorption by both the forms of biomass showed a proportional increase and a plateau was observed at equilibrium concentration of ca. 428.0 mg L^{-1} and 484.1 mg L^{-1} for lyophilized and oven-dried biomass respectively, corresponding to the total metal loading of ca. 79.8 mg Co^{2+} g^{-1} and 25.6 mg Co^{2+} g^{-1} (Fig. 13). The Cu^{2+} sorption by the lyophilized and oven-dried biomass exhibited an almost similar saturating concentration, but the metal loading was found to be much higher at corresponding equilibrium concentrations. At equilibrium concentration, the maximum Cu^{2+} loading capacity was found to be 250 mg Cu^{2+} g^{-1} and 160 mg Cu^{2+} g^{-1} of lyophilized and oven-dried biomass (Fig. 13) respectively. The Zn^{2+} sorption pattern by the lyophilized biomass was found to be hyperbolic and similar to Co^{2+} or Cu^{2+} sorption. Although the saturating equilibrium

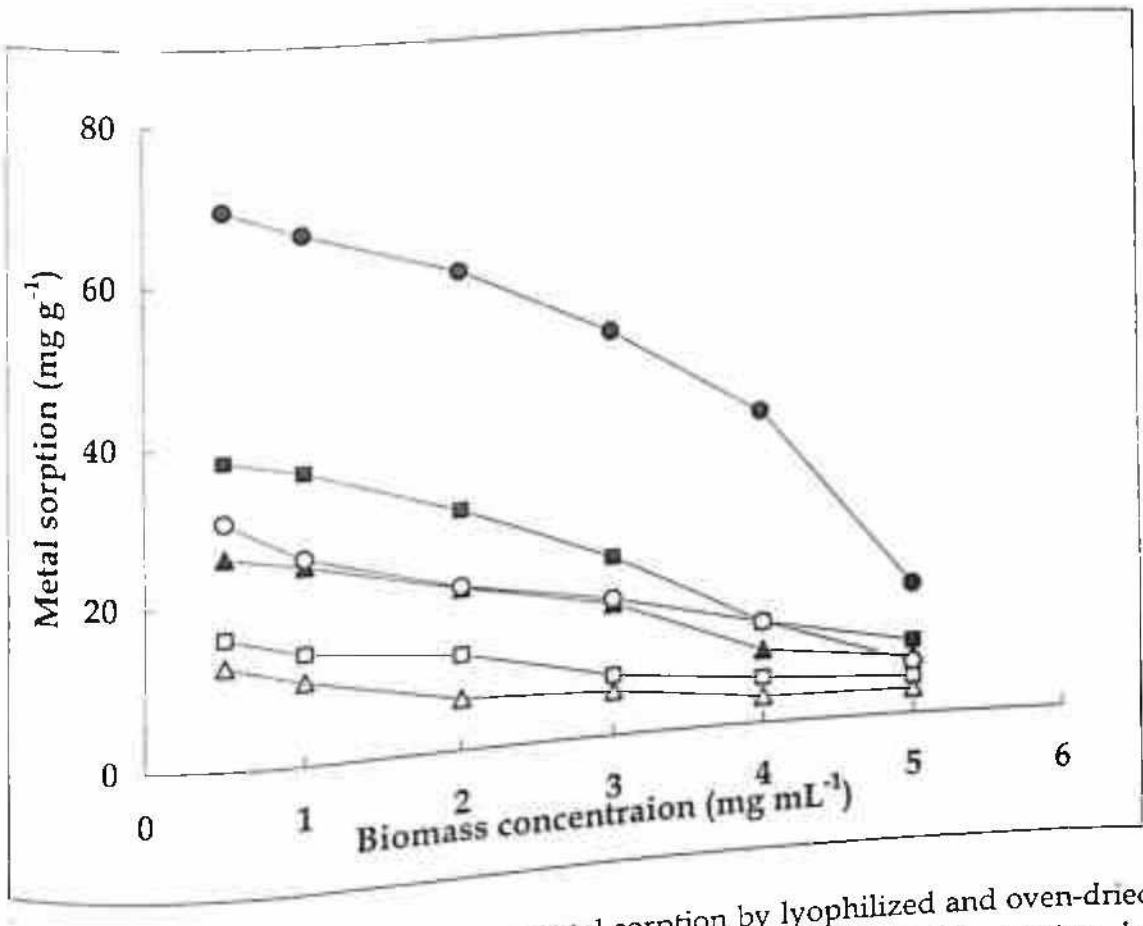

Fig. 12. Effect of biomass density on metal sorption by lyophilized and oven-dried *S.platensis* biomass; Co^{2+} (-▲-), Cu^{2+} (-●-), Zn^{2+} (-■-) sorption by lyophilized biomass; Co^{2+}(-△-), Cu^{2+} (-○-), Zn^{2+} (-□-) sorption by oven-dried biomass.

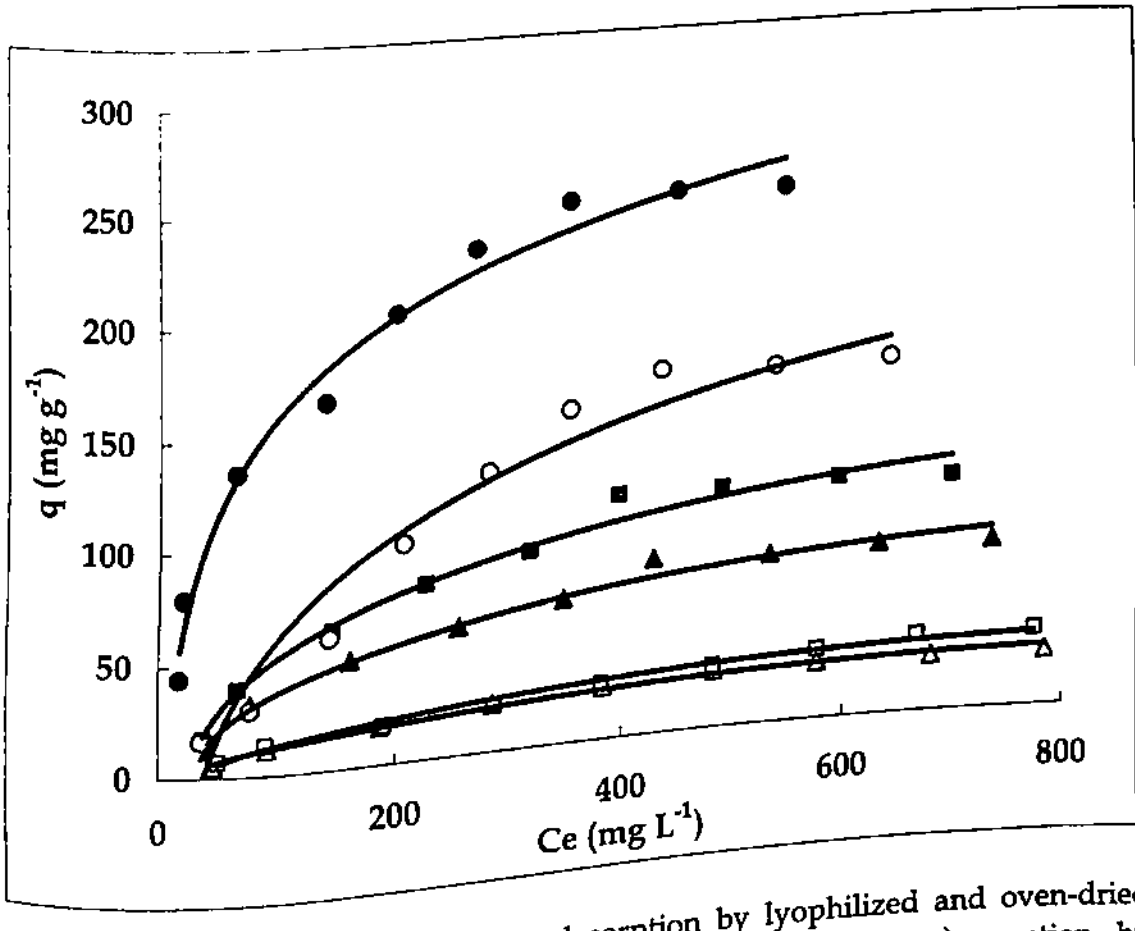

Fig. 13. Equilibrium isotherm of metal sorption by lyophilized and oven-dried *S.platensis* biomass; Co^{2+} (-▲-), Cu^{2+} (-●-), Zn^{2+} (-■-) sorption by lyophilized biomass; Co^{2+}(-△-), Cu^{2+} (-○-), Zn^{2+} (-□-) sorption by oven-dried biomass.

concentration for lyophilized biomass was same as that of Cu^{2+} and Co^{2+} (420 mg L^{-1}), the saturation for oven-dried biomass was attained at much higher concentration (ca. 650 mg L^{-1}). The Zn^{2+} loading was found to be ca. 3.5-fold higher in lyophilized cells as compared to the oven-dried biomass at the respective equilibrium concentration (Fig.13).

3. 3. 1. Linearised sorption isotherms

The sorption relationship between the amount of metal adsorbed by the biomass (q) and the metal concentration in solution (Ce) was further studied by examining the closeness of fit of Langmuir and Freundlich models (Eq-2 and 3). The Langmuir isotherm is based on considering sorption as a chemical phenomenon and is assumed that the forces exerted by chemically unsaturated surface atoms (total number of binding sites B) do not extend further than the diameter of one sorbed molecule and that therefore sorption is restricted to a mono layer. As shown in Table-6, lyophilized *S. platensis* biomass had a maximum adsorption capacity (q^{max}) towards Cu^{2+} (277.7) followed by Zn^{2+} (222.2) and Co^{2+} (169.3). The coefficient 'b', is related to the affinity between the sorbent and sorbate that correlates energy of adsorption through Arrhenius equation, showed low values in general, especially for oven-dried biomass in Cu^{2+} and Zn^{2+} sorption (0.00043 and 0.00058 respectively; Table-6) reflecting the steep initial slope of sorption isotherm and indicating the desirable high affinity of the process (Fig. 14a).

The Freundlich isotherm is an empirical model assuming that sorbents have a suite of sorption sites of different energy and that solute molecules can be adsorbed in an multi layer fashion. The adsorbent capacity 'k' for Co^{2+} sorption by lyophilyzed biomass was found to be 1.33 whereas it was 1.42 with oven-dried biomass (Fig. 14b). Cu^{2+} sorption by lyophilyzed biomass gave rise to 3.40 of 'k' and was 1.14 for oven-dried biomass. Similarly, 'k' value of lyophilized biomass was higher (1.51) as compared to oven-dried

Table-6. Langmuir and Freundlich constants of Co^{2+}, Cu^{2+} and Zn^{2+} sorption by lyophilized and oven-dried *S.platensis* biomass.

Metal	*Biomass type*	*Langmuir constants*			*Freundlich constants*		
		Q^{max} ($mg\,g^{-1}$)	b ($L\,mg^{-1}$)	r^2	k ($mg\,g^{-1}$)	*1/n*	r^2
Co^{2+}	*Lyophilized biomass*	169.5	0.002	0.9483	1.33	0.59	0.8976
	Oven-dried biomass	43.10	0.003	0.9697	1.42	0.38	0.9430
Cu^{2+}	*Lyophilized biomass*	277.7	0.015	0.9456	3.40	0.45	0.9188
	Oven-dried biomass	169.2	0.0006	0.9904	1.14	0.88	0.9611
Zn^{2+}	*Lyophilized biomass*	222.2	0.0024	0.9665	1.51	0.605	0.9229
	Oven-dried biomass	44.2	0.0039	0.9818	1.02	0.57	0.9808

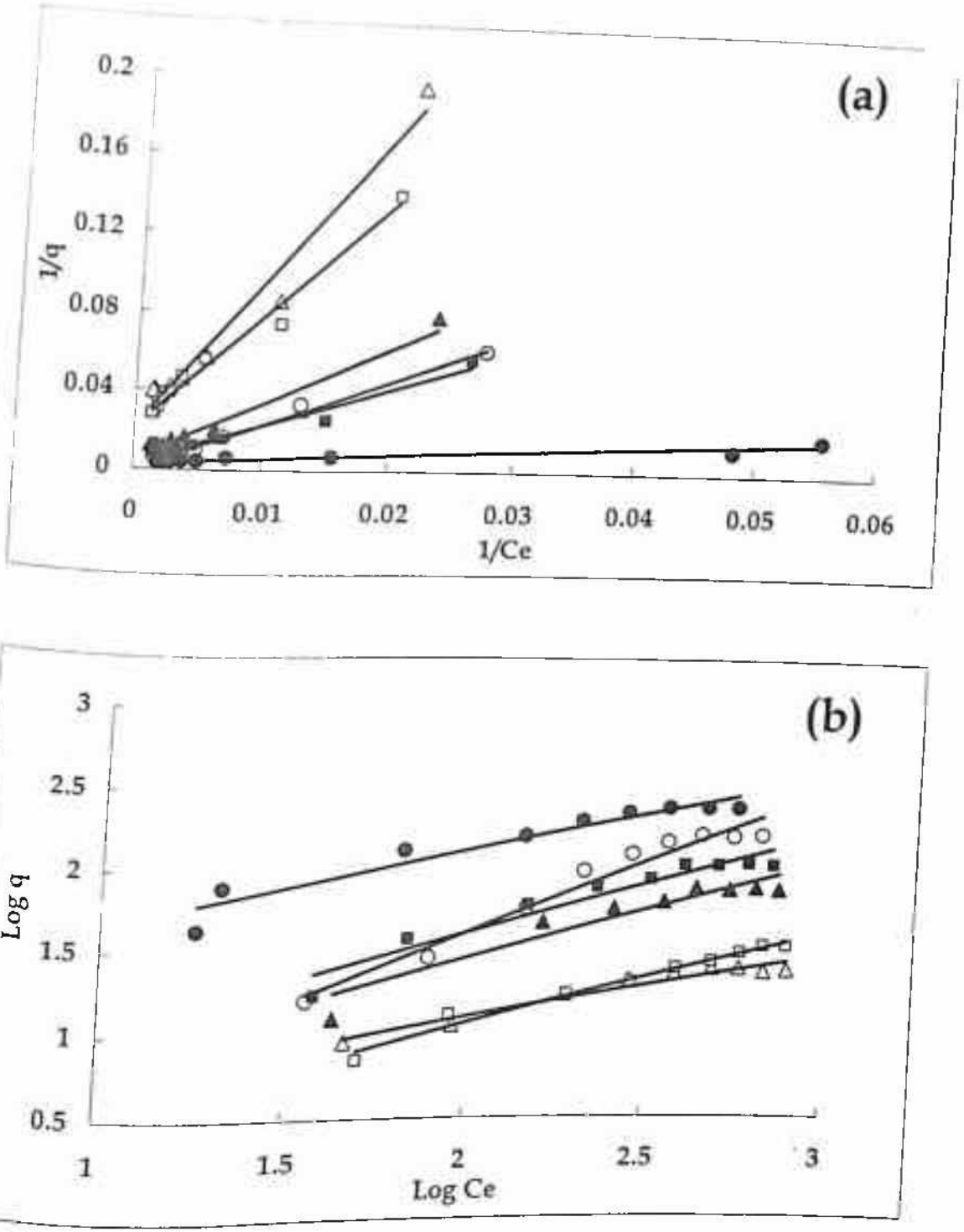

Fig. 14. Linearised (a) Langmuir and (b) Freundlich isotherms of metal sorption by lyophilized and oven-dried *S.platensis* biomass; Co^{2+} (-▲-), Cu^{2+} (-●-), Zn^{2+} (-■-) sorption by lyophilized biomass; Co^{2+}(-△-), Cu^{2+} (-O-), Zn^{2+} (-□-) sorption by oven-dried biomass.

biomass (1.02) for Zn^{2+} sorption. In general a 'k' value of 3.4 was found to be the highest among the observed results that was obtained with lyophilized biomass for Cu^{2+} sorption (Table-6). The sorption intensity '1/n' found to be higher with lyophilyzed biomass for Co^{2+} and Zn^{2+} (0.59 for Co^{2+} and 0.60 for Zn^{2+}) when compared with oven-dried biomass (0.38 for Co^{2+} and 0.57 for Zn^{2+}) where as it was higher for oven-dried biomass in case of Cu^{2+} (0.88) than lyophilized biomass (0.45) as seen in Table-6.

3. 4. Kinetics of metal biosorption

In order to study the kinetics involved in sorption of Co^{2+}, Cu^{2+} or Zn^{2+} by lyophilized and oven-dried biomass of *S.platensis*, the biomass was subjected to 100 mg L^{-1} metal solution for 2 hr. The percent fraction of total metal sorbed as a function of contact time is presented in Fig. 15. The results indicate the biphasic metal sorption phenomenon for both types of biomass studied as in the case of normal biomass. In first phase of metal sorption 74%-77% of the total metal sorbed on to the lyophilized biomass with in ca. 1 min of contact (data not shown) corresponding to a metal loading of ca. 21.2 mg Co^{2+} g^{-1}, 62.2 mg Cu^{2+} g^{-1} and 29.4 mg Zn^{2+} g^{-1} of biomass (Fig. 15). Where as it was 70-73% in the case of oven-dried biomass corresponding to 7.9 mg Co^{2+} g^{-1}, 22.2 mg Cu^{2+} g^{-1} and 11.5 mg Zn^{2+} g^{-1} of biomass. The Secondary residual phase started after ca. 1-2 min at slower uptake phase and was continued up to 2 hr at which equilibrium appeared to be reached for both biomass types used.

3. 5. Metal elution

Regeneration of biomass in a scale-up is a crucial process parameter that influences the operating cost. In present study, the lyophilized or oven-dried biomass pre-exposed to 100 mg L^{-1} of Co^{2+}, Cu^{2+} or Zn^{2+} for 2 hr was treated with different eluants. The general

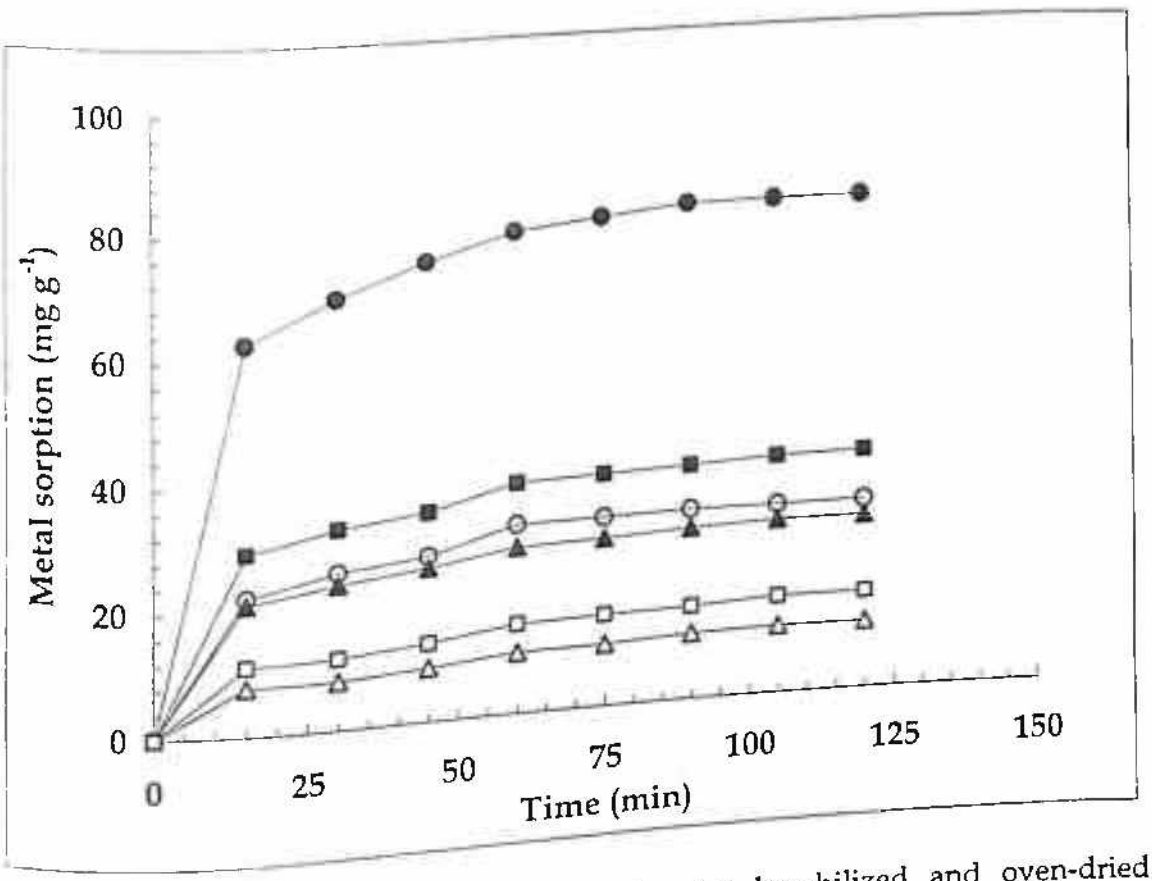

Fig. 15. Kinetics involved in metal sorption by lyophilized and oven-dried *S.platensis* biomass; Co^{2+} (-▲-), Cu^{2+} (-●-), Zn^{2+} (-■-) sorption by lyophilized biomass; Co^{2+}(-△-), Cu^{2+} (-O-), Zn^{2+} (-□-) sorption by oven-dried biomass.

elution efficiency (expressed in percent ratio of metal elution and sorption) of mineral acids was found to be maximum followed by inorganic salts, chelating agent and organic acids. The hydrochloric acid showed maximum elution (ca. 95.5% Co^{2+}, 98.1% Cu^{2+} or 95.6% Zn^{2+} for lyophilized biomass) amongst the mineral acids followed by nitric and sulfuric acid. Amongst the organic acids citric acid showed more elution than acetic acid. The Ca $(NO_3)_2$ in the category of inorganic salts, showed maximum elution of Cu^{2+} ca. 95.1%, whereas $CaCl_2$ was found to be more efficient in Co^{2+} (93.6%) and Zn^{2+} (95.1%) elution from metal treated lyophilized biomass (Table-7). Sodium EDTA showed above 90% elution of bound metal from the biomass. The oven-dried biomass also followed a similar trend of metal recovery with different type of eluants (Table-8). A comparison of metal elution from metal loaded lyophilized and oven-dried biomass showed that the metal recovery with similar eluants was more efficient for the former condition.

3. 6. Effect of temperature

The sorption of Co^{2+}, Cu^{2+} and Zn^{2+} by lyophilized or oven-dried *S.platensis* biomass was studied over a temperature range of 10°C-50°C. The data indicate that there was no significant difference in metal sorption values of three metals when temperature rose from 10°C to 30°C. However ca. 9% and 13% reduction was observed at 40°C for lyophilized and oven-dried biomass, respectively. It was followed by further reduction in metal sorption when temperature increased to 50°C corresponding to 16% and 19% for lyophilized and oven-dried biomass respectively (Fig. 16).

3. 7. Effect of co-ions

The presence of co-ions (Cd^{+2}, Ni^{+2}, Co^{2+}, Cu^{2+} and Zn^{2+}) on the uptake of target metal ions (Co^{2+}, Cu^{2+} and Zn^{2+}) at equimolar concentration condition in *S. platensis* biomass against control is shown in Table-9. The results clearly indicate that the equimolar concentration

Table-7. Elution of Co^{2+}, Cu^{2+} and Zn^{2+} by lyophilized *S.platensis* biomass.

Elution reagent	*Amount of metal eluted into reagent (%)*		
	Co^{2+}	Cu^{2+}	Zn^{2+}
Distilled deionized water	0.03	0.1	0.06
H_2SO_4 (0.1M)	94.1	95.8	94.9
HCl (0.1M)	95.5	98.1	95.6
HNO_3 (0.1M)	95.0	98.0	95.1
Acetic acid (0.1M)	77.7	72.0	77.1
Citric acid (0.1M)	78.9	64.0	79.3
NaCl (10 mM)	66.1	7.2	69.3
Na_2CO_3 (10 mM)	83.1	88.0	81.3
NaHCo3 (10 mM)	85.6	79.0	87.1
$CaCl_2$ (10 mM)	93.6	76.2	95.1
$CaCO_3$ (10 mM)	90.1	81.2	94.9
$Ca(NO_3)_2$ (10 mM)	91.1	95.1	90.6
Na_2EDTA (10 mM)	92.6	98.0	90.0

Table-8. Elution of Co^{2+}, Cu^{2+} and Zn^{2+} by oven-dried *S.platensis* biomass.

Elution reagent	*Amount of metal eluted into reagent (%)*		
	Co^{2+}	Cu^{2+}	Zn^{2+}
Distilled deionized water	0.03	0.1	0.01
H_2SO_4 (0.1M)	95.0	93.2	94.1
HCl (0.1M)	95.1	95.6	94.9
HNO_3 (0.1M)	95.0	95.0	94.0
Acetic acid (0.1M)	74.6	71.9	75.1
Citric acid (0.1M)	76.3	68.6	77.3
NaCl (10 mM)	65.3	23.6	68.3
Na_2CO_3 (10 mM)	81.1	82.1	80.1
NaHCo3 (10 mM)	82.1	79.0	85.6
$CaCl_2$ (10 mM)	91.3	79.1	94.6
$CaCO_3$ (10 mM)	90.0	81.9	93.9
$Ca(NO_3)_2$ (10 mM)	90.0	94.0	89.1
Na_2EDTA (10 mM)	90.0	91.1	90.1

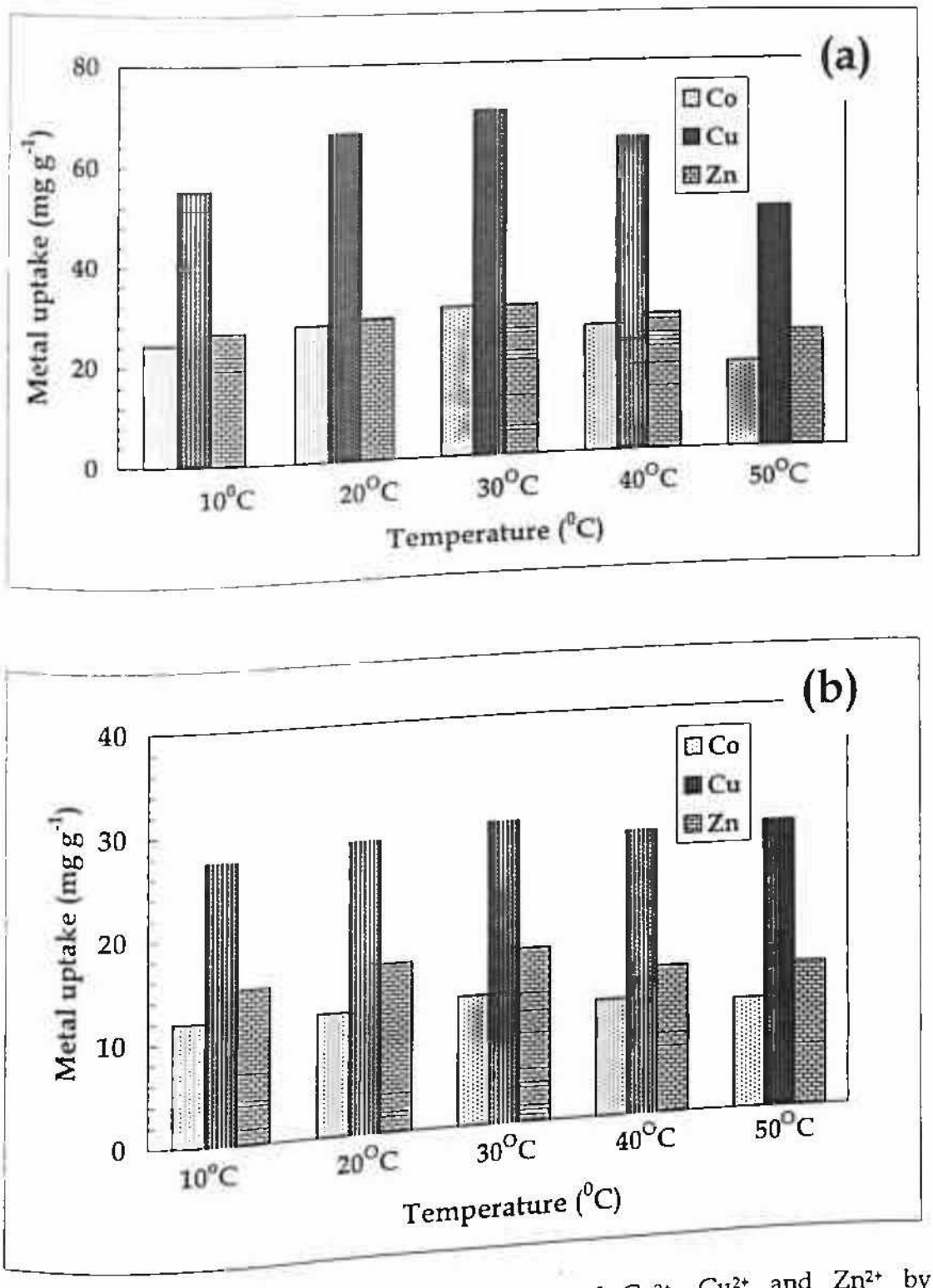

Fig. 16. Effect of temperature on sorption of Co^{2+}, Cu^{2+} and Zn^{2+} by (a) lyophilized biomass and (b) oven-dried biomass of *S. platensis*.

Table-9. Effect of cations on sorption of Co^{2+}, Cu^{2+} and Zn^{2+} by lyophilized or oven-dried biomass of *S. platensis* in bi-metallic solutions.

Additional Cations	*Reduction in metal sorption of Co^{2+} (%)*		*Reduction in metal sorption Cu^{2+} (%)*		*Reduction in metal sorption Zn^{2+} (%)*	
	Lyophilized	*Oven-dried*	*Lyophilized*	*Oven-dried*	*Lyophilized*	*Oven-dried*
Control	0.0	0.0	0.0	0.0	0.0	0.0
Co^{2+}	--	--	4.6	5.7	2.6	8.7
Ni^{2+}	34.6	44.8	3.9	6.0	4.9	5.8
Cu^{2+}	47.2	54.5	--	--	12.8	22.9
Zn^{2+}	29.2	54.9	6.6	8.8	--	--
Cd^{2+}	8.8	9.3	4.1	4.2	8.5	10.9

of Cu^{2+} showed the maximum inhibition of Co^{2+} and Zn^{2+} uptake in both lyophilized and oven-dried biomass. The Co^{+2} sorption was reduced by 47.2% in the lyophilized biomass and 54.5% in oven-dried biomass in presence of equimolar concentration of Cu^{2+}. Similarly, it inhibited ca. 13% and 23% of Zn^{2+} sorption under lyophilized and oven-dried conditions respectively. Similarly, the presence of Co^{2+} showed inhibitory effect on Cu^{2+} (4.6% for lyophilized and 5.7% for oven-dried biomass) and Zn^{2+} (2.6% for lyophilized and 8.7% for oven-dried biomass) sorption. Maximum inhibition in Co^{2+} uptake by Zn^{2+} was found to be ca. 55% for oven-dried biomass and it was 47.2% under lyophilized condition. Further, it was observed that the presence of Ni^{+2} and Cd^{+2} was also inhibitory towards uptake of Co^{2+}, Cu^{2+} and Zn^{2+} by both forms of biomass. The overall inhibition efficiency of different cations can be summarized as

	Co^{2+}	Cu^{2+}	Zn^{2+}
Lyophilyzed	Cu > Ni > Zn > Cd	Zn > Co > Cd > Ni	Cu > Cd > Ni > Co
Oven-dried	Cu = Zn > Ni > Cd	Zn > Ni > Co > Cd	Zn > Cd > Co > Ni

4. Immobilization of *Spirulina platensis* and its recharacterisation for metal uptake

The physical form of biomass used in any biosorption process is an important factor in a metal recovery system. While cells in suspension can provide valuable information in laboratory experimentation, in the more rigorous industrial applications free microbial biomass poses major disadvantages. Thus the immobilized or pelleted biomass is generally used in packed bed or fluidized bed reactors. Keeping this in view, the studies were conducted to examine the metal uptake process using two types of entrapment (alginates and polyacrylamide gels) techniques.

4. 1. Biomass loading into calcium alginate (Ca-Alg) and polyacrylamide gel (PAG) matrices, their stability and metal uptake

In order to examine the biomass loading, calcium alginate (Ca-Alg) or polyacrylamide gel matrices were loaded with various concentrations of normal or lyophilized biomass and examined for their stability and metal uptake. Results clearly indicate that the alginate beads with normal biomass were sufficiently stable in their mechanical strength up to a loading capacity of 8.3% on dry weight basis, whereas beads with lyophilized biomass were quite stable even at a loading of 14.3% of their dry weight. A higher density of biomass in the bead led to cell leakage into reaction mixture as a few colonies appeared on agar plates at the biomass loading beyond 8.3% and 14.3% for normal and lyophilized biomass respectively (data not shown). The value of maximum metal uptake was also found to be optimal at 14.3% of lyophilized biomass loading whereas it was 8.3% with normal biomass (Fig. 17). On the other hand, PAG cubes containing normal biomass were quite stable in their mechanical strength until they were loaded to their 11.1% on dry weight basis whereas cubes loaded with lyophilized biomass were found to be stable even at biomass loading of ca. 43% on dry weight basis. A higher density of biomass in the cube showed leakage of cells into the reaction vessel. Maximum metal uptake was found at 27.9% of lyophilized biomass loading whereas it was 11.1% with normal biomass (Fig. 18). Therefore, 27.9% lyophilized biomass loading in polyacylamide gel matrix was employed in subsequent experiments due to its stability and optimal metal uptake. Further, Ca-Alg and PAG matrices without biomass (empty beads/cubes) also exhibited metal uptake. While, maximum metal uptake by Ca-Alg found to be higher (33.1 mg Co^{2+} g^{-1}, 47.3 mg Cu^{2+} g^{-1} and 45.0 mg Zn^{2+} g^{-1}, w/w), the PAG cubes showed lesser metal

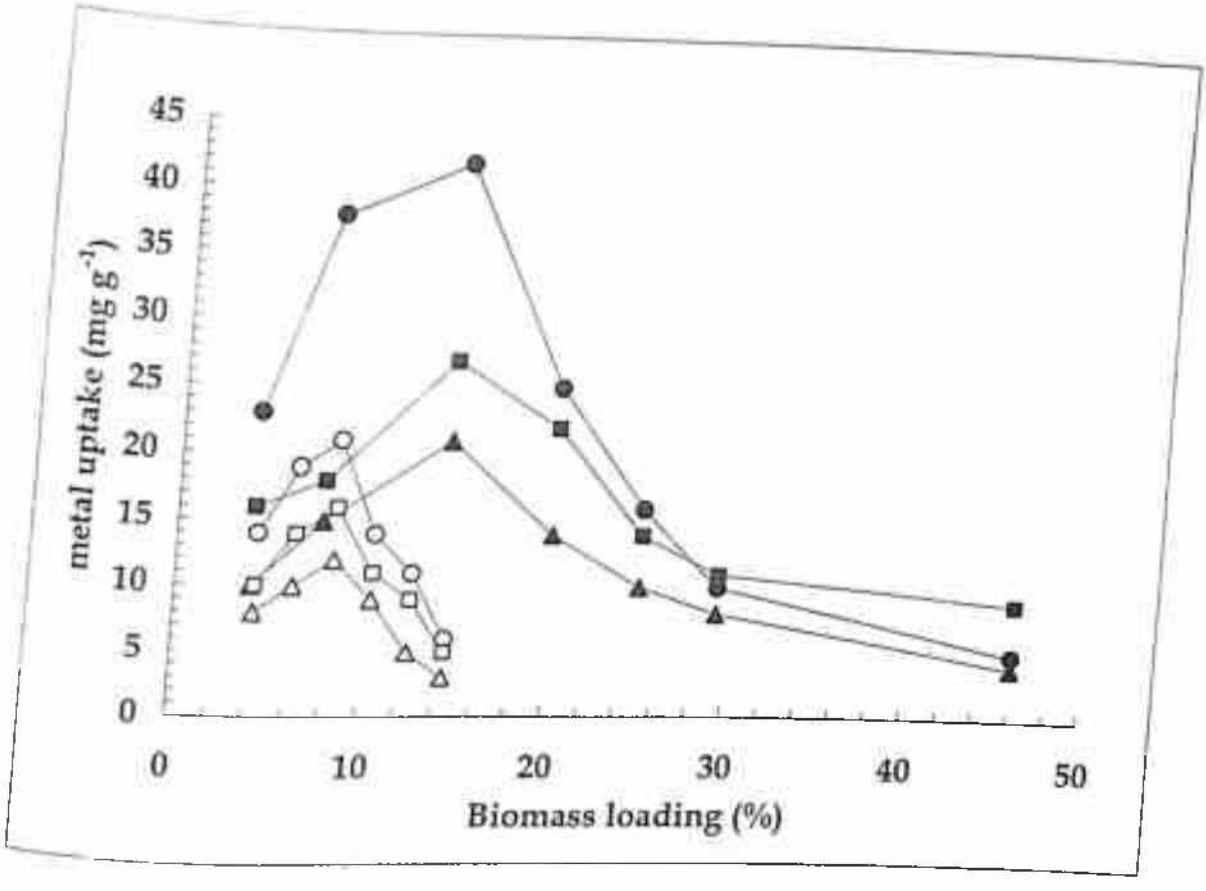

Fig. 17. Metal uptake by different concentration of normal and lyophilized *S.platensis* biomass into Ca-Alg matrix; Co^{2+} (-△-), Cu^{2+} (-○-), Zn^{2+} (-□-) uptake by bead having normal biomass; Co^{2+} (-▲-), Cu^{2+} (-●-), Zn^{2+} (-■-) uptake by bead having lyophilized biomass.

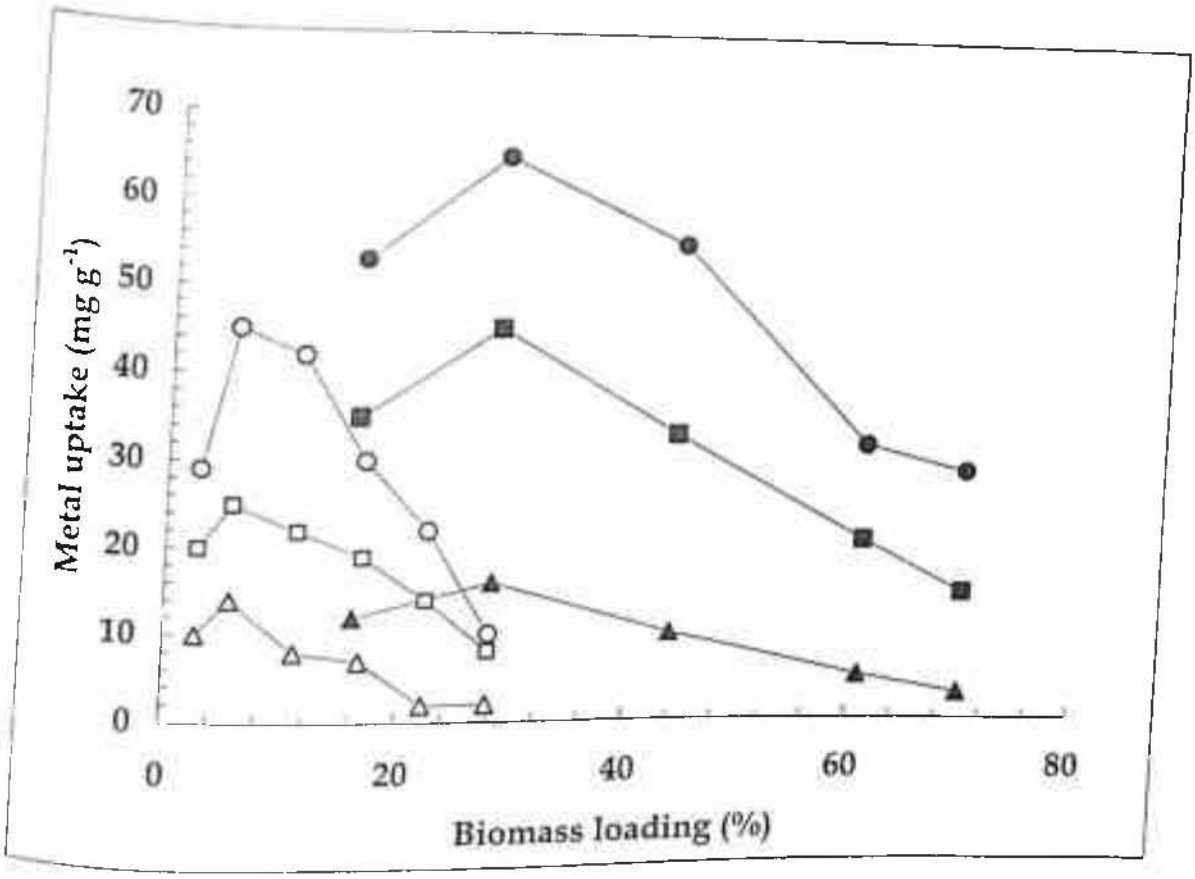

Fig. 18. Metal uptake by different concentration of normal and lyophilized *S.platensis* biomass into PAG matrix; Co^{2+} (-△-), Cu^{2+} (-○-), Zn^{2+} (-□-) uptake by cube having normal biomass; Co^{2+} (-▲-), Cu^{2+} (-●-), Zn^{2+} (-■-) uptake by cube having lyophilized biomass.

uptake (3.1 mg Co^{2+} g^{-1}, 5.4 mg Cu^{2+} g^{-1} and 4.9 mg Zn^{2+} g^{-1}, w/w). The values presented here are obtained after subtracting the uptake values of empty beads/cubes.

4. 2. Effect of pH

The metal uptake pattern by the PAG immobilized lyophilized *S.platensis* biomass showed a regular increase in uptake with increasing pH in the acidic range reaching a maximum value of 13.3 mg Co^{2+} g^{-1}, 65.6 mg Cu^{2+} g^{-1} and 18.1 mg Zn^{2+} g^{-1} biomass at pH 6.0. The metal uptake declined sharply at pH 6.5 (Fig. 19). Hence, investigation above pH 6.5 was not conducted because of possible metal precipitation.

4. 3. Equilibrium metal sorption

Biosorption equilibrium isotherms were developed to examine the sorption performance of PAG immobilized *S.platensis* biomass by conducting the experiments at varying initial metal concentrations as observed for free cells earlier. For the lowest Co^{2+} equilibrium concentration (28.9 mg L^{-1}), the sorption was 23.1 Co^{2+} g^{-1} of biomass. The sorption capacity was almost doubled to 47.4 mg Co^{2+} g^{-1} of biomass when the equilibrium concentration rose to 237.7 mg L^{-1}. The sorption showed a proportional increase and a plateau was observed at equilibrium concentration of 426.3 mg L^{-1} corresponding to total metal loading of 78.6 mg Co^{2+} $g^{-1}$of biomass (Fig. 20). Similarly, the Cu^{2+} sorption was found to be 41.6 mg Cu^{2+} g^{-1} for the lowest equilibrium concentration of 11.0 mg L^{-1}. The sorption capacity was increased to 120.9 mg Cu^{2+} g^{-1} (3-fold) when the equilibrium concentration rose to 83.6 mg L^{-1}, attaining saturation at equilibrium concentration of 253.2 mg L^{-1} corresponding to a sorption of 250.1 mg Cu^{2+} g^{-1} of biomass (Fig. 20). The Zn^{2+} sorption also showed a similar trend leading to 112.8 mg Zn^{2+} g^{-1} of biomass at the biomass saturation with the equilibrium concentration of 391.0 mg L^{-1} (Fig. 20).

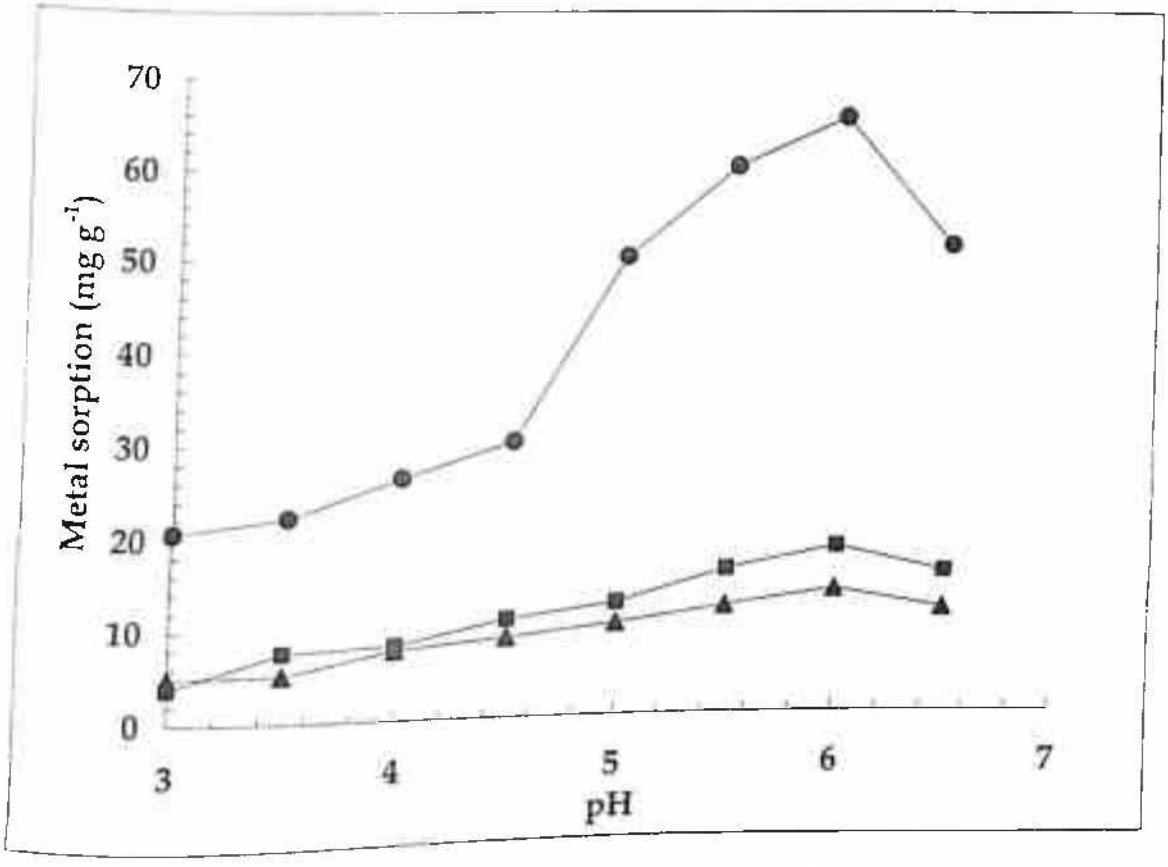

Fig. 19. Effect of pH on metal sorption by PAG immobilized *S.platensis* biomass; Co^{2+} (-▲-), Cu^{2+} (-●-), Zn^{2+} (-■-).

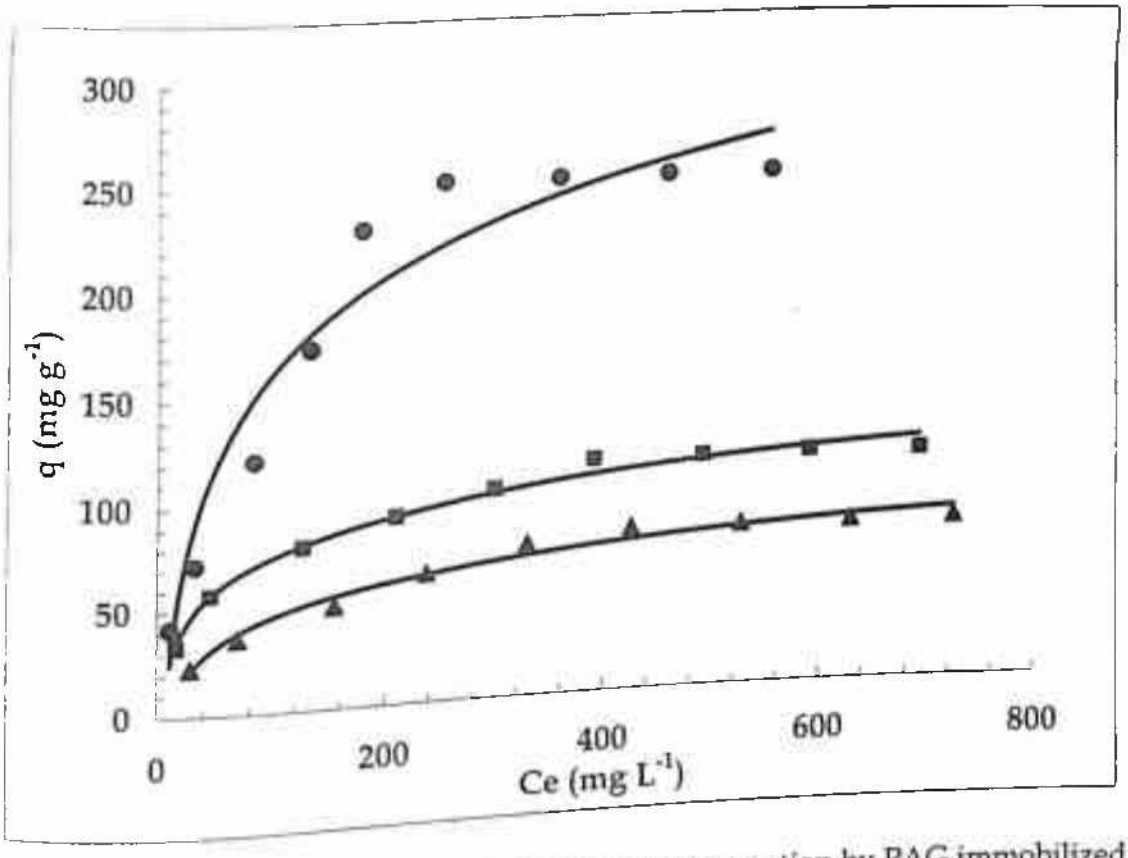

Fig. 20. Equilibrium isotherm of Co^{2+}, Cu^{2+} and Zn^{2+} sorption by PAG immobilized *S.platensis*; Co^{2+} (-▲-), Cu^{2+} (-●-), Zn^{2+} (-■-).

4. 3. 1. Linearised sorption isotherms

The sorption relationship was further characterized by examining the closeness of fit of Langmuir and Freundlich models (Eq-2 and 3) as seen for free cells (Fig.21a-b). The constant 'q_{max}' in Langmuir model, representing the highest possible sorbate sorption, found to be highest for Cu^{2+} (250.0) followed by Zn^{2+} (116.3) and Co^{2+} (85.5); Table-10. The coefficient 'b' was found to be lowest for Co^{2+} (0.012) followed by Cu^{2+} (0.017) and Zn^{2+} (0.023). Freundlich constant 'k' (adsorbent capacity) was highest for Cu^{2+} (3.12) followed by Co^{2+} (2.28) and Zn^{2+} (1.03). Conversely the sorption intensity ('1/n') was found to be highest for Zn^{2+} (0.55) followed by Cu^{2+} (0.50) and Co^{2+} (0.39).

4. 4. Kinetics of metal sorption

The metal sorption by PAG immobilized *S.platensis* biomass indicates that like free cells the process involves two distinct phases of an initial rapid sorption of metal followed by secondary slow and residual metal sorption phase. Initial rapid phase was completed with in 1-2 min (data not shown) of contact time followed by a secondary residual phase, which continued for 2 hr leading to saturation. The first phase of rapid binding contributed 67-70% of the total metal sorption in all metals studied (Fig. 22).

4. 5. Metal elution

To elute the bound metal from *S.platensis* biomass entrapped in PAG squares, various reagents like aqueous solutions of mineral acids, organic acids, inorganic acids and chelating agents were tested for efficiency in eluting the bound metal. The mineral acids were found to be superior in their of elution efficiency followed by inorganic salts, chelating agent and organic acids. Among mineral acids, the nitric acid showed maximum elution (varying from 92% - 95% efficiency) followed by hydrochloric and sulfuric acid. The Ca $(NO_3)_2$ showed maximum elution

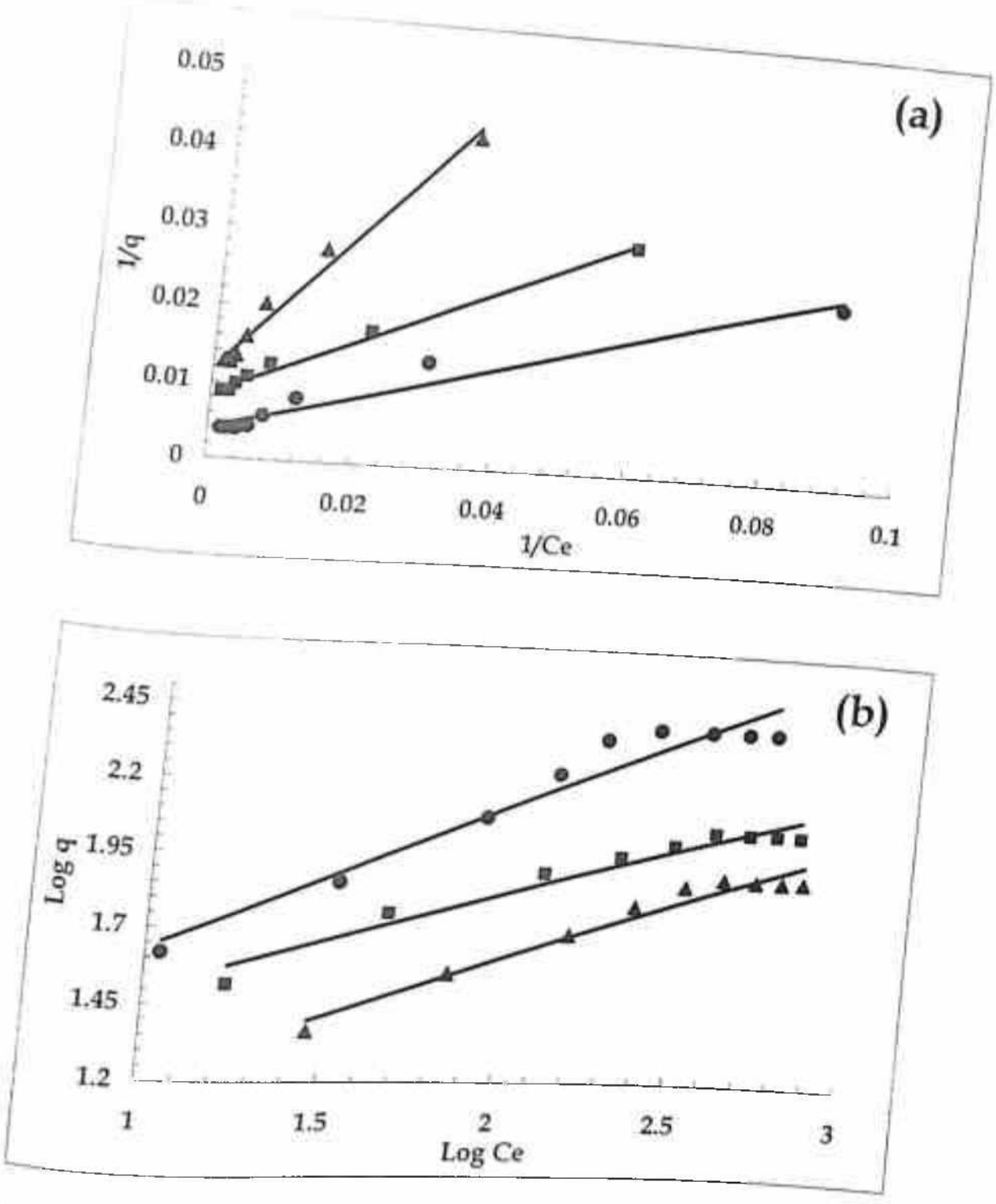

Fig. 21. Linearised (a) Langmuir and (b) Freundlich isotherms of Co^{2+}, Cu^{2+} and Zn^{2+} by PAG immobilized *S.platensis* biomass; Co^{2+} (-▲-), Cu^{2+} (-●-), Zn^{2+} (-■-).

Table-10. Langmuir and Freundlich constants of Co^{2+}, Cu^{2+} and Zn^{2+} sorption by PAG immobilized *S.platensis* biomass.

Metal	*Langmuir constants*			*Freundlich constants*		
	q^{max} ($mg\,\bar{g}^{1}$)	*b* ($L\,m\bar{g}^{1}$)	r^2	*k* ($mg\,\bar{g}^{1}$)	*1/n*	r^2
Co^{2+}	106.4	0.007	0.9647	2.23	0.41	0.9582
Cu^{2+}	250.0	0.0172	0.9634	3.12	0.50	0.9477
Zn^{2+}	123.4	0.0322	0.9634	4.62	0.201	0.9455

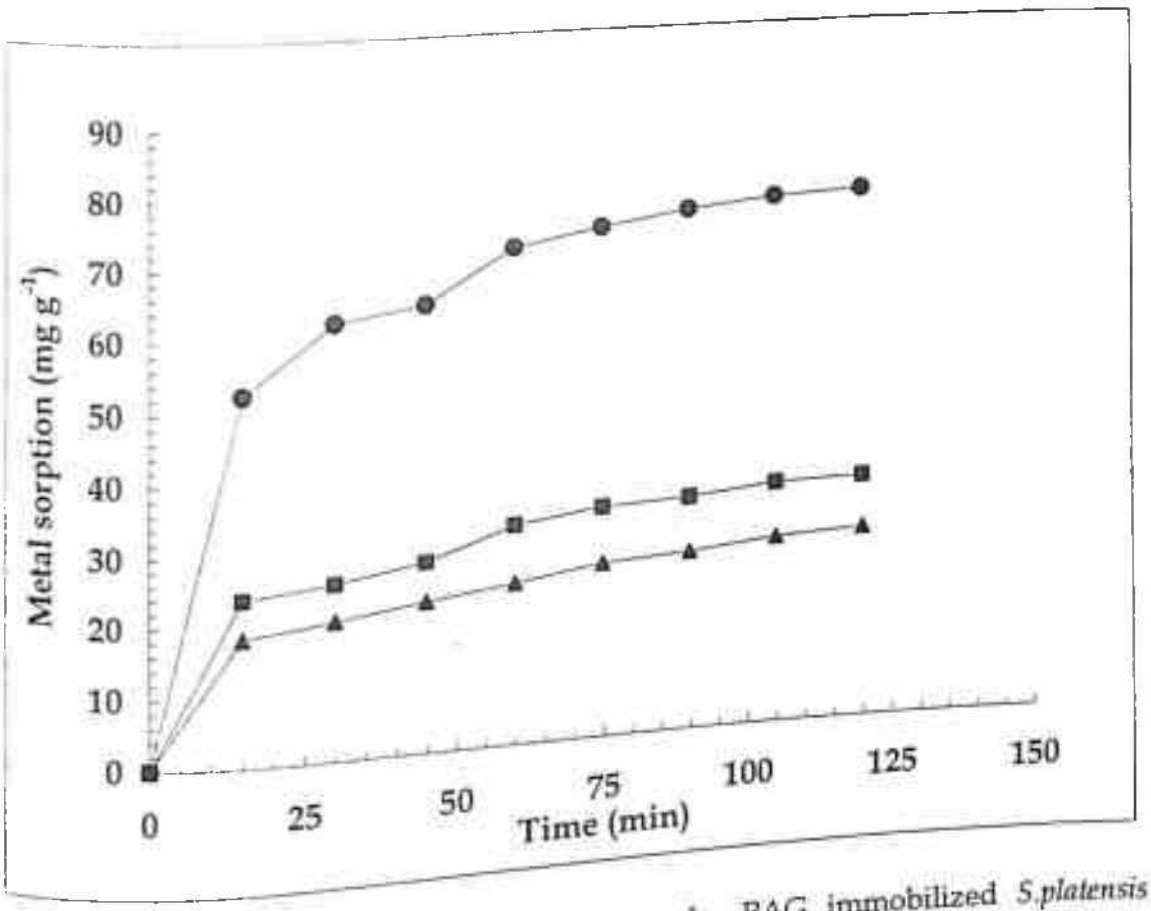

Fig. 22. Kinetics involved in metal sorption by PAG immobilized *S.platensis* biomass; Co^{2+} (-▲-), Cu^{2+} (-●-), Zn^{2+} (-■-).

of Cu^{2+} (94.3%), whereas $CaCl_2$ was found to be more efficient in Co^{2+} (91.6%) and Zn^{2+} (94.0%) elution from metal treated biomass (Table-11). Sodium EDTA showed above 90% elution of bound metal from the biomass.

4. 6. Multiple cycles of metal sorption and elution

In order to exploit the biomass for continuous metal removal and recovery in turn biomass regeneration in a batch system, ten cycles of sorption and elution was performed. PAG immobilized *S.platensis* biomass showed high efficiency (in its sorption and elution) for at least seven continuous cycles with 96.0% to 98.0% and 95.0% to 99% of sorption and elution of the supplied metal. The efficiency of sorption and elution was reduced after the seventh cycle (Fig. 23). At the tenth cycle, while the sorption of Co^{2+}, Cu^{2+} or Zn^{2+} was 42.0%-47.0%, the elution was found to be in the range of 51.0%-56.0% respectively.

4. 7. Effect of temperature

The results of the sorption of Co^{2+}, Cu^{2+} or Zn^{2+} by PAG immobilized *S.platensis* biomass over the temperature range (10^0C-50^0C) indicate that there was no significant difference in metal sorption values of these metals when temperature was elevated from 10^0C to 40^0C followed by a decrease beyond 50^0C like those of the free cells. A maximum of 40% reduction was observed in Cu^{2+} sorption at 50^0C followed by 38% of Zn^{2+} and 23% of Co^{2+} sorption at the same temperature (Fig 24).

4. 8. Effect of co-ions

In nature, the toxic metallic ions are rarely found in isolation, thus the studies involving various combinations of metal ions would give insite in to metal-metal vis-a-vis metal-microbe interactions. The effect of co-ion (Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} or Fe^{3+}) on the sorption of the target metal ion (Co^{2+}, Cu^{2+} or Zn^{2+}) by PAG immobilized *S.platensis*

Table-11. Elution of Co^{2+}, Cu^{2+} and Zn^{2+} by PAG immobilized *S.platensis* biomass.

Elution reagent	*Amount of metal eluted into reagent (%)*		
	Co^{2+}	Cu^{2+}	Zn^{2+}
Distilled deionized water	0.00	0.03	0.00
H_2SO_4 (0.1M)	91.3	92.3	92.1
HCl (0.1M)	92.6	94.3	92.1
HNO_3 (0.1M)	92.9	94.6	92.6
Acetic acid (0.1M)	65.6	79.3	70.1
Citric acid (0.1M)	70.1	69.1	75.3
NaCl (10mM)	52.1	69.3	60.12
Na_2CO_3 (10mM)	71.6	85.6	77.1
NaHCo3 (10mM)	75.1	75.7	85.1
$CaCl_2$ (10mM)	91.6	70.1	94.0
$CaCO_3$ (10mM)	89.1	79.1	91.2
$Ca(NO_3)_2$ (10mM)	89.9	94.3	88.1
Na_2EDTA (10mM)	84.1	92.9	85.1

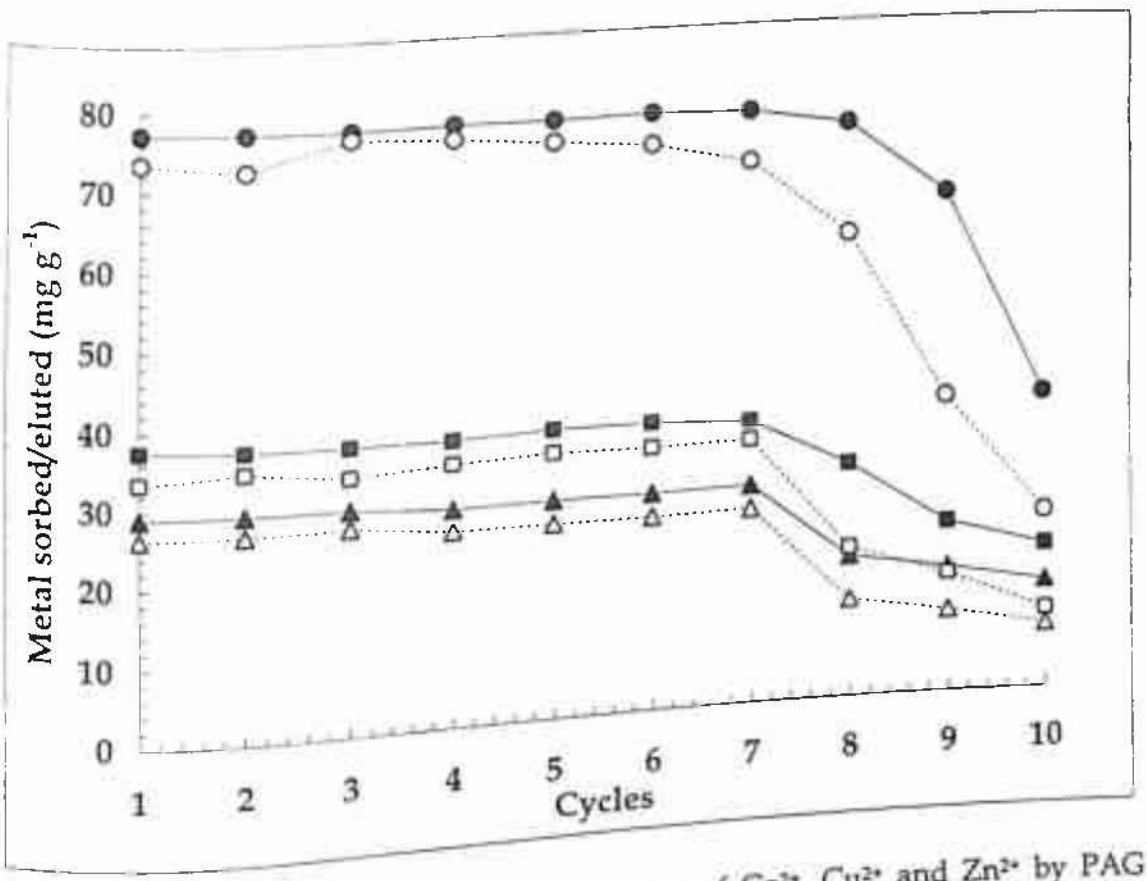

Fig. 23. Multiple cycles of sorption and elution of Co^{2+}, Cu^{2+} and Zn^{2+} by PAG immobilized *S.platensis* biomass; Co^{2+} (-▲-), Cu^{2+} (-●-), Zn^{2+} (-■-) sorption, Co^{2+} (-△-), Cu^{2+} (-○-), Zn^{2+} (-□-) elution.

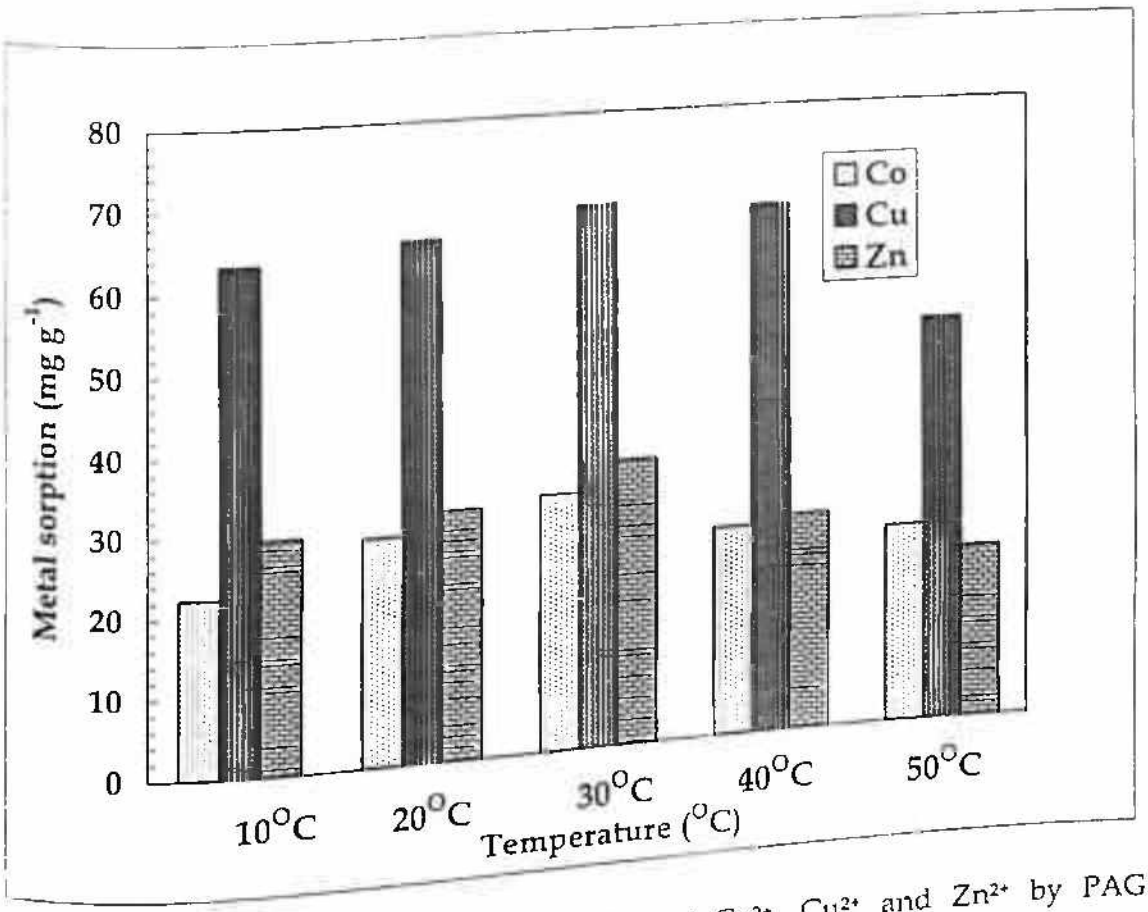

Fig. 24. Effect of temperature on sorption of Co^{2+}, Cu^{2+} and Zn^{2+} by PAG immobilized *S.platensis* biomass.

biomass was examined under increasing co-ion concentration while keeping target ion concentration constant. As the concentration of co-ion increased from 200 mg L^{-1} to 1400 mg L^{-1}, the uptake of target metal ion (at its saturation concentration) was found to be decreased in all cases examined (Fig. 25-27). The reduction in uptake of Co^{2+} (500 mg L^{-1}) was found as 11.6% when Cu^{2+} concentration was 200 mg L^{-1} but it increased to 39.5% when Cu^{2+} concentration was 1000 mg L^{-1} in the system. However, reduction was found to be 40.0% when Cu^{2+} concentration increased to 1400 mg L^{-1} (Fig. 25). Similarly, as the Ni^{2+} concentration increased from 200 mg L^{-1} to 1400 mg L^{-1} the Co^{2+} uptake was found to decrease from 6.3% to 17.9% and was 18.0% when Ni^{2+} concentration increased to 1400 mg L^{-1}. Conversely, Zn^{2+} and Cd^{2+} were also found to reduce Co^{2+} uptake to a total of 33.1% and 23.6% respectively when their concentration was 1400 mg L^{-1} (Fig. 25). Among all co-ions tested, Fe^{3+} was found to be most inhibitory. The reduction in Co^{2+} sorption was found to be 21.1% when Fe^{3+} ion concentration was 200 mg L^{-1} and increased to 60.3% when concentration rose to 400 mg L^{-1}. Thereafter there was no increase in reduction of Co^{2+} sorption even at 1400 mg L^{-1} of Fe^{3+} concentration (Fig. 25). Similarly, the maximum reduction in sorption of Cu^{2+} and Zn^{2+} was observed with Fe^{3+} when the concentration of Fe^{3+} was 400 mg L^{-1} above which there was no change in reduction. The reduction by the rest of the co-ions was found to be 15-20% in the case of Cu^{2+} sorption (Fig. 26) whereas it was 51% for Zn^{2+} sorption (Fig. 27).

5. Continuous removal and recovery of heavy metals and radionuclides using up-flow packed bed columnar reactor

5. 1. Optimization of process parameters for removal and recovery of $Cu^{2+}$and Zn^{2+}

The packed bed columnar reactor containing PAG immobilized *S.platensis* biomass was optimized for removal and recovery of Cu^{2+} or Zn^{2+}. The data presented here is obtained

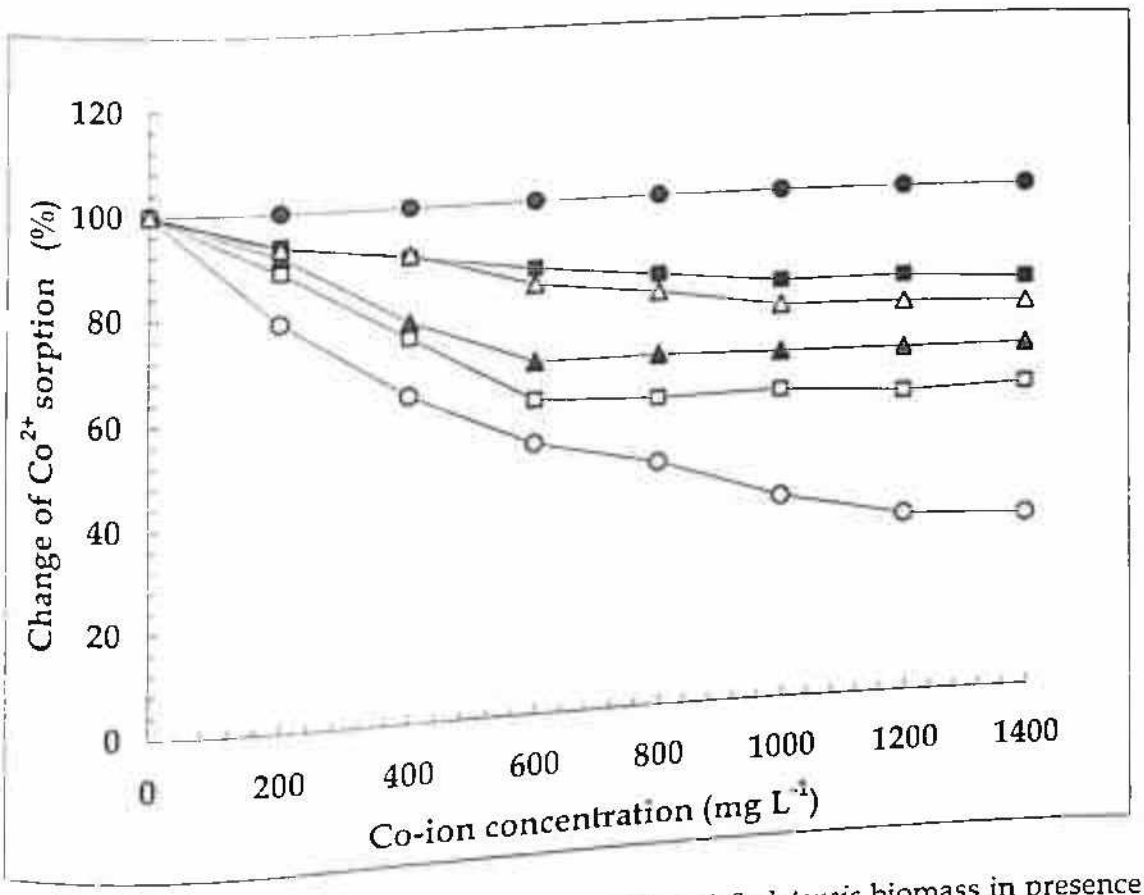

Fig. 25. Percent Co^{2+} sorption by PAG immobilized *S.platensis* biomass in presence of Fe^{3+} (-○-), Ni^{2+} (-■-), Cu^{2+} (-□-), Zn^{2+} (-▲-) and Cd^{2+} (-△-); The maximum Co^{2+} sorption (q_{max} = 78.6 mg g^{-1}) in the absence of co-ion was taken as control Co^{2+} (-● -).

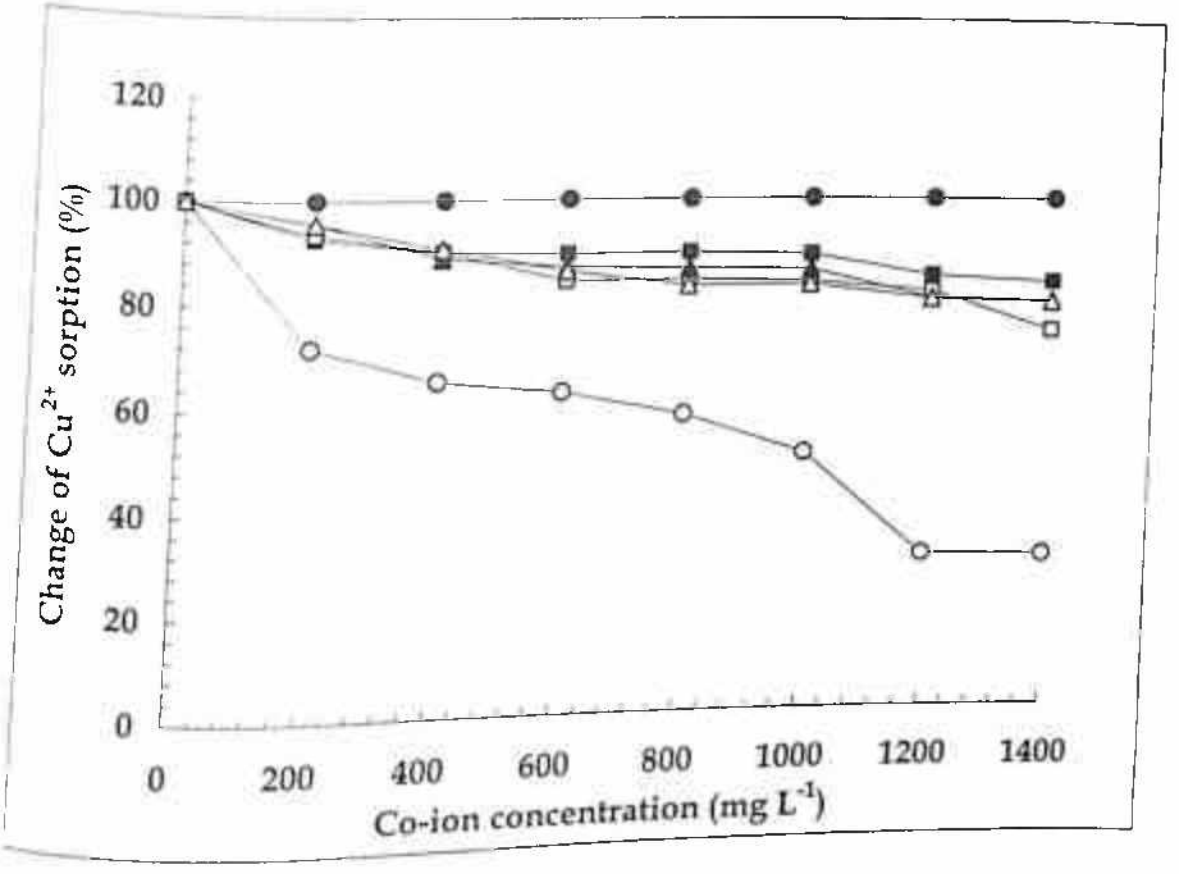

Fig. 26. Percent Cu^{2+} sorption by PAG immobilized *S.platensis* biomass in presence of Fe^{3+} (-O-), Ni^{2+} (-■-), Co^{2+} (-□-), Zn^{2+} (-▲-) and Cd^{2+} (-△-); The maximum Cu^{2+} sorption (q_{max} = 250.1 mg g^{-1}) in the absence of co-ion was taken as control Cu^{2+} (-●-).

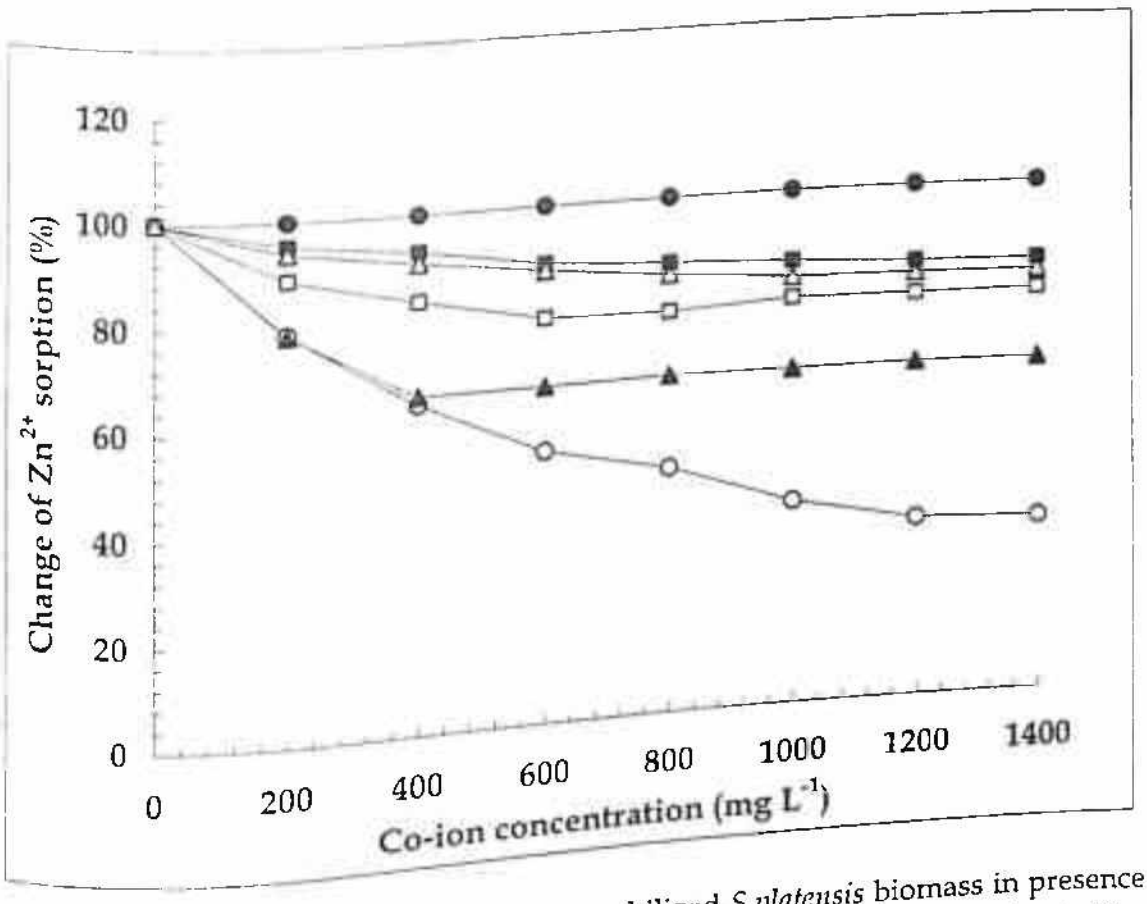

Fig. 27. Percent Zn^{2+} sorption by PAG immobilized *S.platensis* biomass in presence of Fe^{3+} (-O-), Ni^{2+} (-■-), Co^{2+} (-□-), Cu^{2+} (-▲-) and Cd^{2+} (-△-); The maximum Zn^{2+} sorption (q_{max} = 112.8 mg g^{-1}) in the absence of co-ion was taken as control Zn^{2+} (-●-).

after subtracting the sorption and recovery values shown by control cubes. The breakthrough curves for Cu^{2+} solution (0.1 mg mL^{-1}) pumped at the flow rate of 2 mL min^{-1} into the reactor, showed the appearance of Cu^{2+} in the reactor effluent (breakthrough) only after pumping 32.4 L of solution. The amount of Cu^{2+} in the reactor effluent showed a steady increase with time attaining a maximum value at 41.6 L of influent pumping suggesting saturation of the system. A doubling of flow rate (4 mL min^{-1}) showed an early appearance of breakthrough at 30.2 L and saturation after 39.5 L of pumping. The breakthrough and saturation volume further decreased with increasing flow rate (Fig. 28).

The recovery of metal from Cu^{2+} loaded PAG cubes were studied by pumping 10 mM Ca $(NO_3)_2$ (as standardized for batch studies) into the column at varying flow rate (2-8 mL min^{-1}) immediately after metal sorption. While, 315 mL of eluant was required for complete elution of Cu^{2+} at the flow rate of 2 mL min^{-1}, the eluant requirement decreased with increasing flow rate of the eluant (Fig. 29). Such results are expressed in terms of volume reduction (a ratio of influent volume for saturation to the volume of eluant required for complete elution) and concentration factor obtained after one cycle of sorption and elution (Table-12). A maximum of 104-fold volume reduction was observed at the residence time of 4.6 min (flow rate = 6 mL min^{-1}) for Cu^{2+}. The same residence time showed a 109-fold increase in concentration factor which is significant for any metal recovery process. The increase or decrease in residence time was found to decrease both these parameters.

Conversely, Zn^{2+} removal and recovery using the similar packed bed columnar reactor also followed the similar pattern observed for Cu^{2+} removal and recovery showing optimal efficiency at 6 mL min^{-1} of influent flow rate (Fig. 30). At this flow rate,

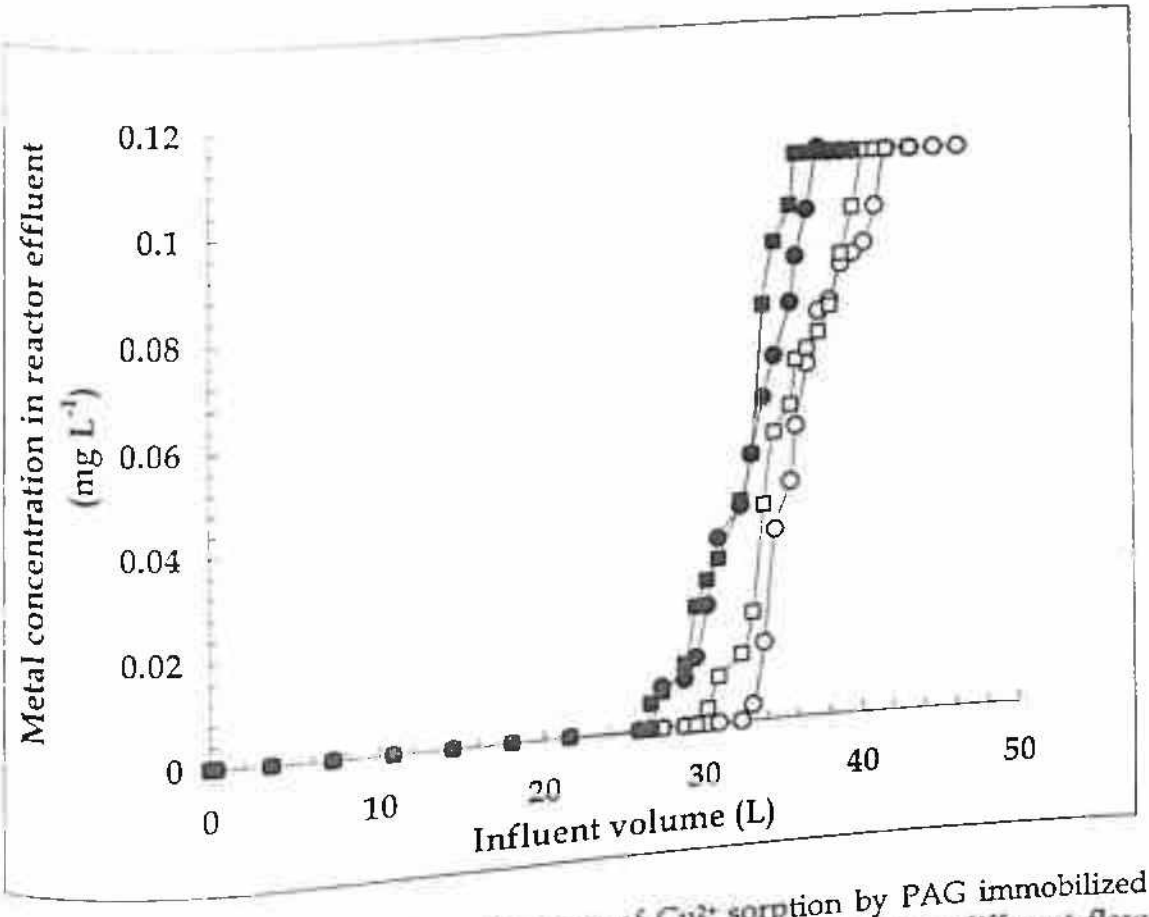

Fig. 28. Experimental breakthrough curve of Cu^{2+} sorption by PAG immobilized *S.platensis* biomass in a packed bed columnar reactor at different flow rates; 2 mL min^{-1} (-○-), 4 mL min^{-1} (-□-), 6 mL min^{-1} (-●-), 8 mL min^{-1} (-■-). The pH of influent 5.9 ± 0.13.

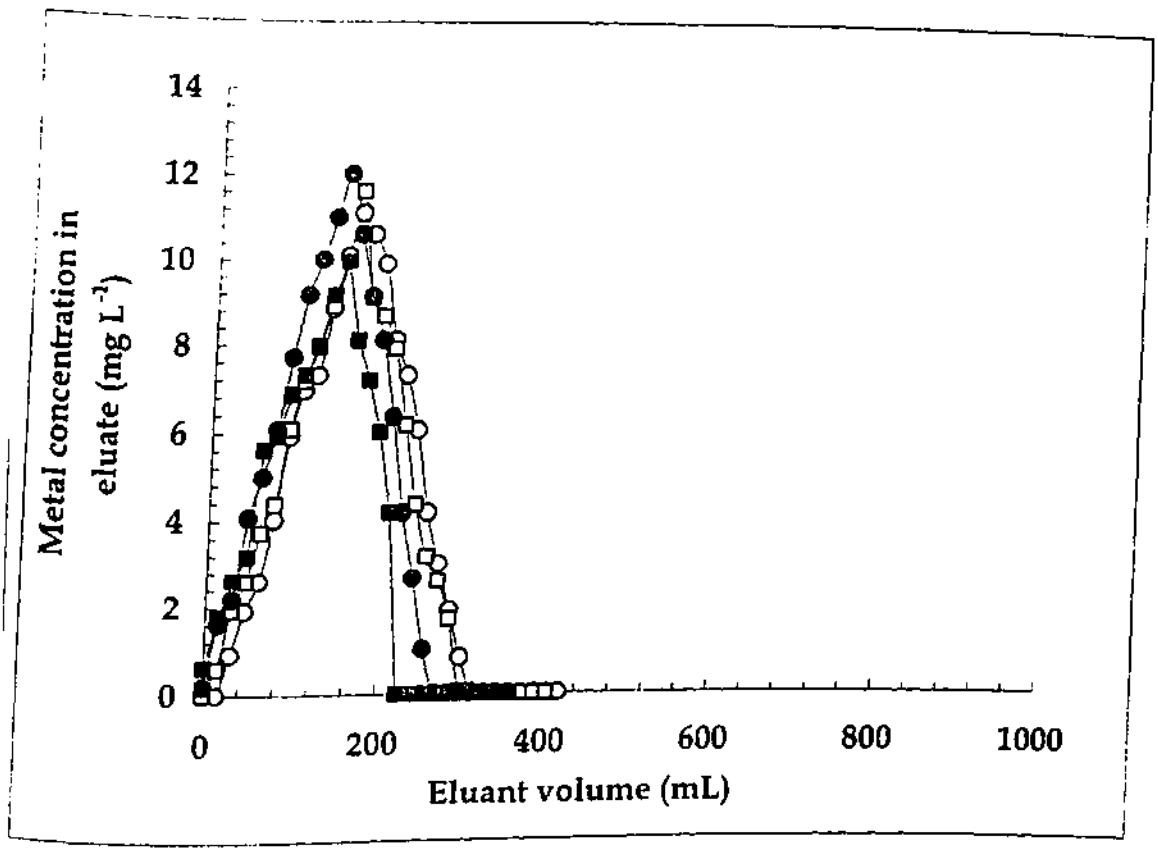

Fig. 29. Elution of Cu^{2+} from PAG immobilized *S.platensis* biomass in packed bed columnar reactor at different flow rates; 2 mL min^{-1} (-O-), 4 mL min^{-1} (-□-), 6 mL min^{-1}(-●-), 8 mL min^{-1} (-■-); The pH of eluant 6.03± 0.03 and pH of metal rich nitrate solution 5.8± 0.12.

Table-12. Performance efficiency of the column packed with PAG immobilized *S.platensis* biomass.

Residence time (min)	*Volume reduction (fold)*		*Concentration factor (fold)*	
	Cu^{2+}	Zn^{2+}	Cu^{2+}	Zn^{2+}
14	102.8	29.6	101.1	64.0
7	98.4	32.6	105.7	72.5
4.6	104.4	38.3	109.3	83.4
3.5	101.6	37.6	90.8	85.7

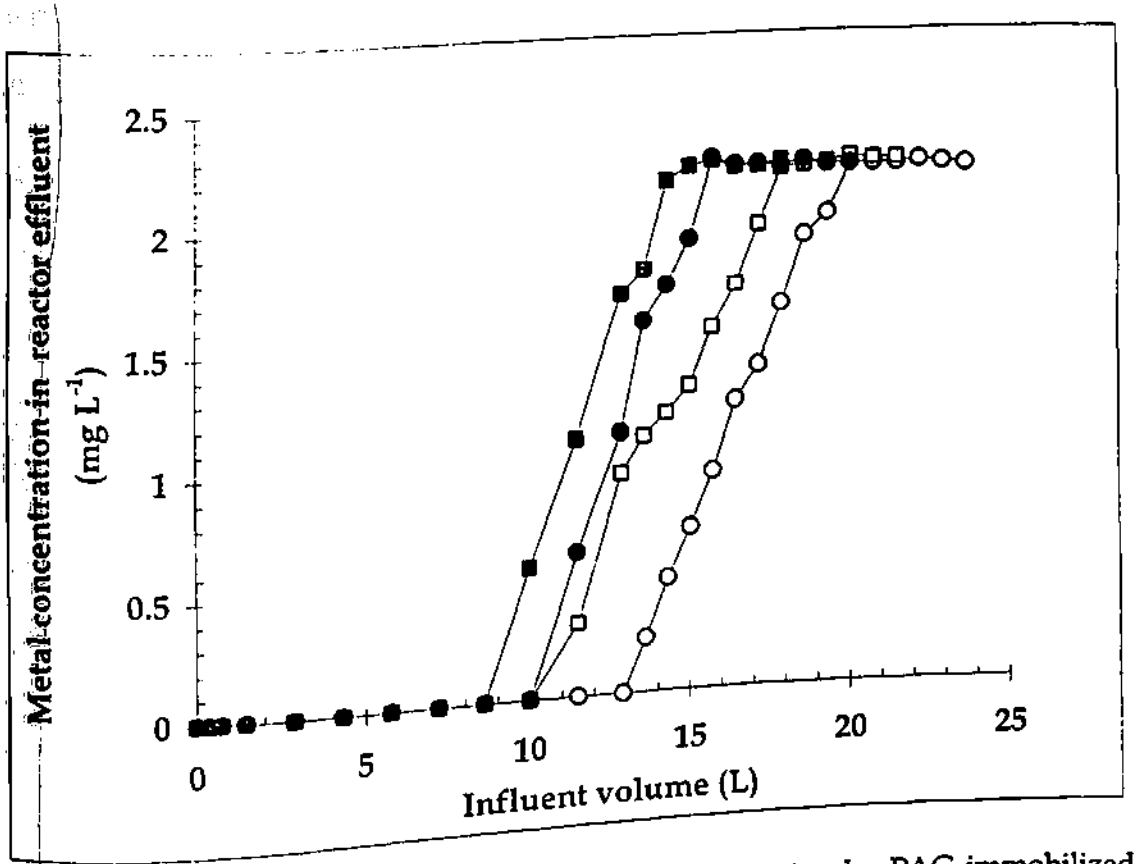

Fig. 30. Experimental breakthrough curve of Zn^{2+} sorption by PAG immobilized *S.platensis* biomass in a packed bed columnar reactor at different flow rates; 2 mL min^{-1} (-○-), 4 mL min^{-1} (-□-), 6 mL min^{-1} (-●-), 8 mL min^{-1} (-■-). The pH of influent 5.9 ± 0.1.

breakthrough appeared at ca. 11.5 L, which attained saturation at ca. 15.8 L of influent volume. Approximately 300 mL of 10 mM $CaCl_2$ was required for 95% of Zn^{2+} recovery at the same flow rate leading to ca. 35-fold volume reduction and 83-fold concentration factor (Fig. 31; Table-12). Thus the residence time of 4.6 min (equivalent to the flow rate of 6 mL min^{-1}) was used in subsequent studies involving simulated waste.

5. 2. Multiple cycles of metal removal and recovery

For the efficient use of PAG immobilized *S.platensis* biomass, the multiple cycles of metal sorption and elution was carried out in the columnar reactor. The Cu^{2+} solution (0.1 mg L^{-1}) was pumped through the column for 112 hr at the flow rate of 6 mL min^{-1}. For elution of Cu^{2+}, Ca $(NO_3)_2$ was pumped into the metal loaded column at 6 mL min^{-1} flow rate. The same column was used for the ten subsequent cycles of metal sorption and elution. The first seven cycles showed almost similar value of Cu^{2+} sorption and elution followed by 30% reduction in both the processes in subsequent cycles. The metal present in PAG immobilized *S.platensis* biomass over multiple cycles of loading and elution with respect to time is presented in Fig. 32a. The volume reduction and concentration factor calculated for ten cycles of Cu^{2+} sorption and elution showed 101-fold reduction in volume and 97-fold increase in concentration factor for first seven cycles, followed by 30% reduction in both the parameters in subsequent cycles (Fig.32b).

5. 3. Removal and recovery of Cu^{2+} and Zn^{2+} from simulated electro-plating industrial wastewater

After the optimization of residence time with flow rate for the packed bed reactor, the sorption of Cu^{2+} and Zn^{2+} was further studied in simulated electro-plating industrial wastewater containing Cu^{2+} and Zn^{2+} in the ratio of 1:22. The Cu^{2+} appeared in the reactor effluent after passing the ca. 26 L of influent volume and the bed was saturated at ca. 33 L

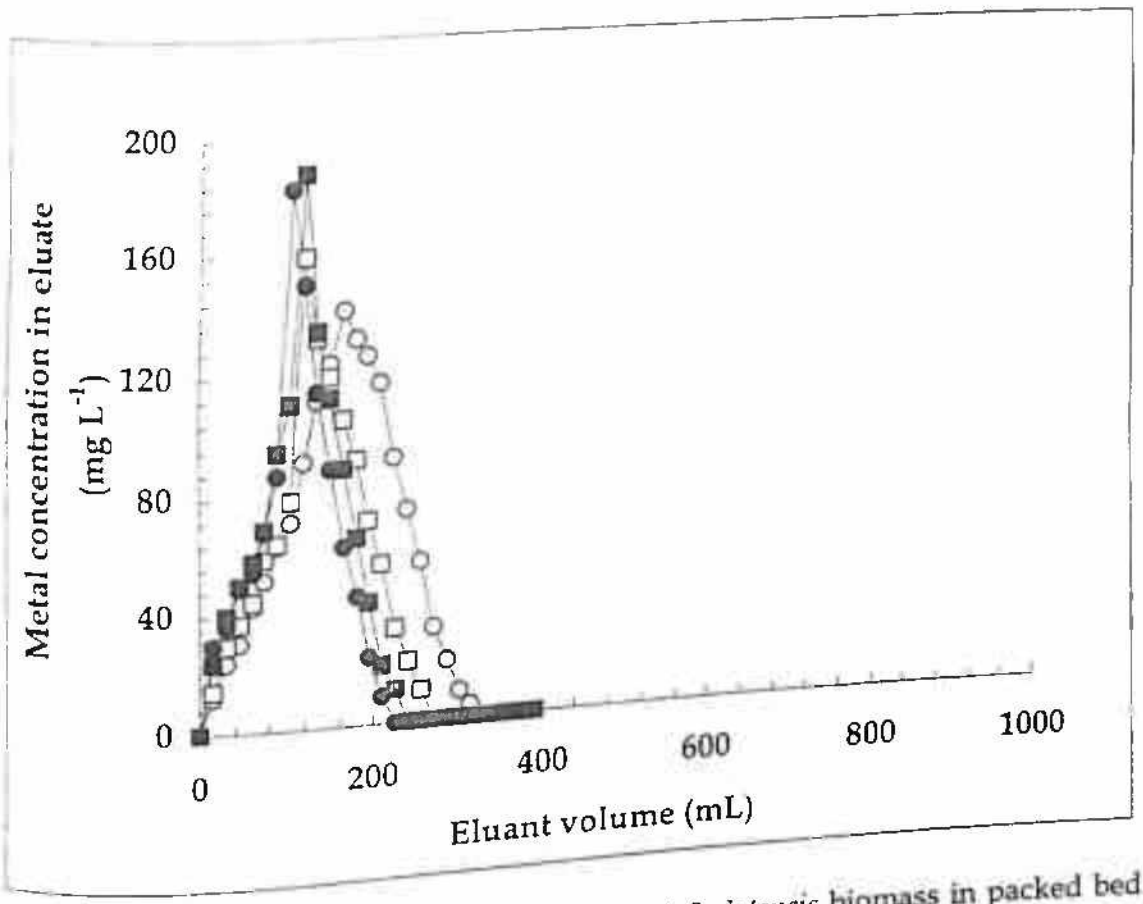

Fig. 31. Elution of Zn^{2+} from PAG immobilized *S.platensis* biomass in packed bed columnar reactor at different flow rates; 2 mL min^{-1} (-O-), 4 mL min^{-1} (-□-), 6 mL min^{-1} (-●-), 8 mL min^{-1} (-■-); The pH of eluant 6.02± 0.03 and pH of metal rich nitrate solution 5.7± 0.12.

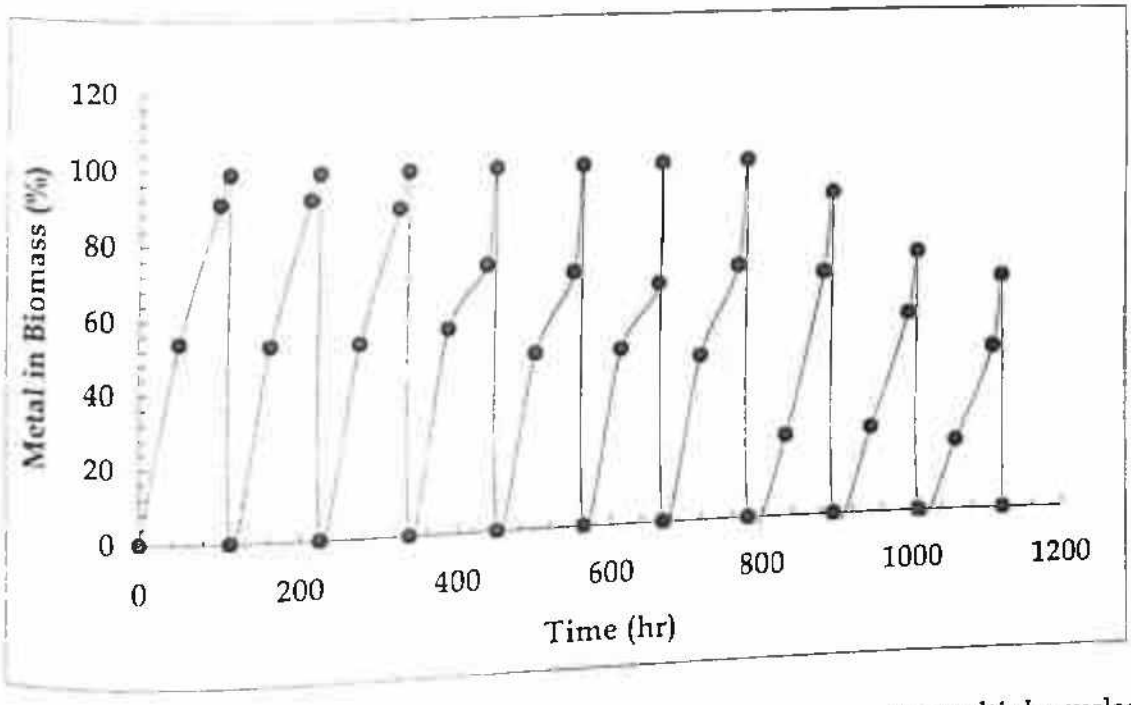

Fig.32a. Sorption and elution of Cu^{2+} by *S.platensis* biomass over multiple cycles using packed bed columnar reactor.

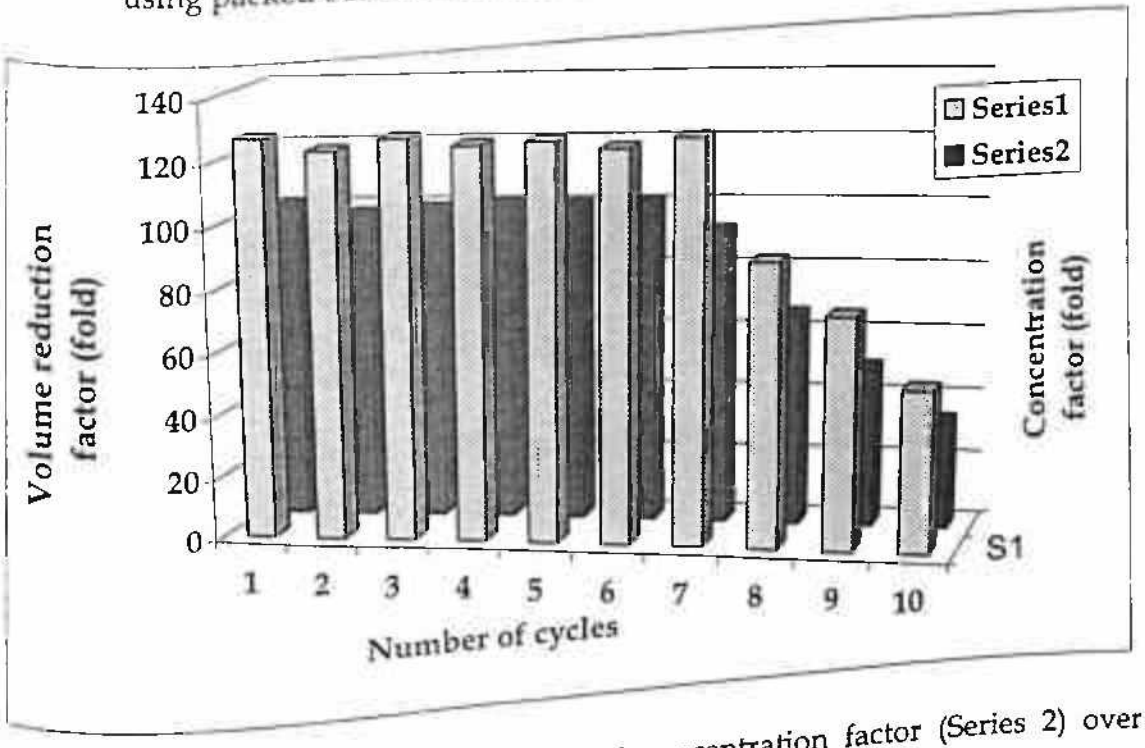

Fig. 32b. Volume reduction (Series 1) and concentration factor (Series 2) over multiple cycles of Cu^{2+} sorption and elution.

of feed (Fig. 33). Whereas, Zn^{2+} showed early appearance at ca. 5.7 L of influent pumping and showed saturation at ca. 15.7 L (Fig. 34). For the recovery of Cu^{2+} or Zn^{2+} in the loaded PAG squares, 10 mM Ca $(NO_3)_2$ or $CaCl_2$ were used separately. A 300 mL Ca $(NO_3)_2$ was required for the complete recovery of Cu^{2+} from the loaded biomass, whereas 420 mL of $CaCl_2$ was required for Zn^{2+} recovery (Fig. 35 and 36). The values for volume reduction and concentration factor for this sorption and elution experiment are shown in Table-13.

5. 4. Radioactive ^{65}Zn and $^{85+89}Sr$ removal

The column containing PAG immobilized *S.platensis* was examined for removal of ^{65}Zn (0.1 mg L^{-1}) and ^{90}Sr (2 mg L^{-1}) radionuclides separately as they are most radiotoxic fission products present in radioactive waste. The breakthrough curve obtained for ^{65}Zn sorption at 6 mL min^{-1} flow rate is as shown in Fig. 37 and it is almost similar to that obtained for non-radioactive Zn^{2+} sorption earlier (Fig. 30). The ^{65}Zn appeared in the reactor effluent after passing ca. 11.3 L of influent and the biomass showed saturation after ca. 16 L of influent passed through. In case of $^{85+89}Sr$ sorption, the process showed early breakthrough at 1.9 L of influent pumping with the same flow rate of 6 mL min^{-1}. However, the biomass showed slow saturation requiring 9.5 L of influent pumping (Fig.37).

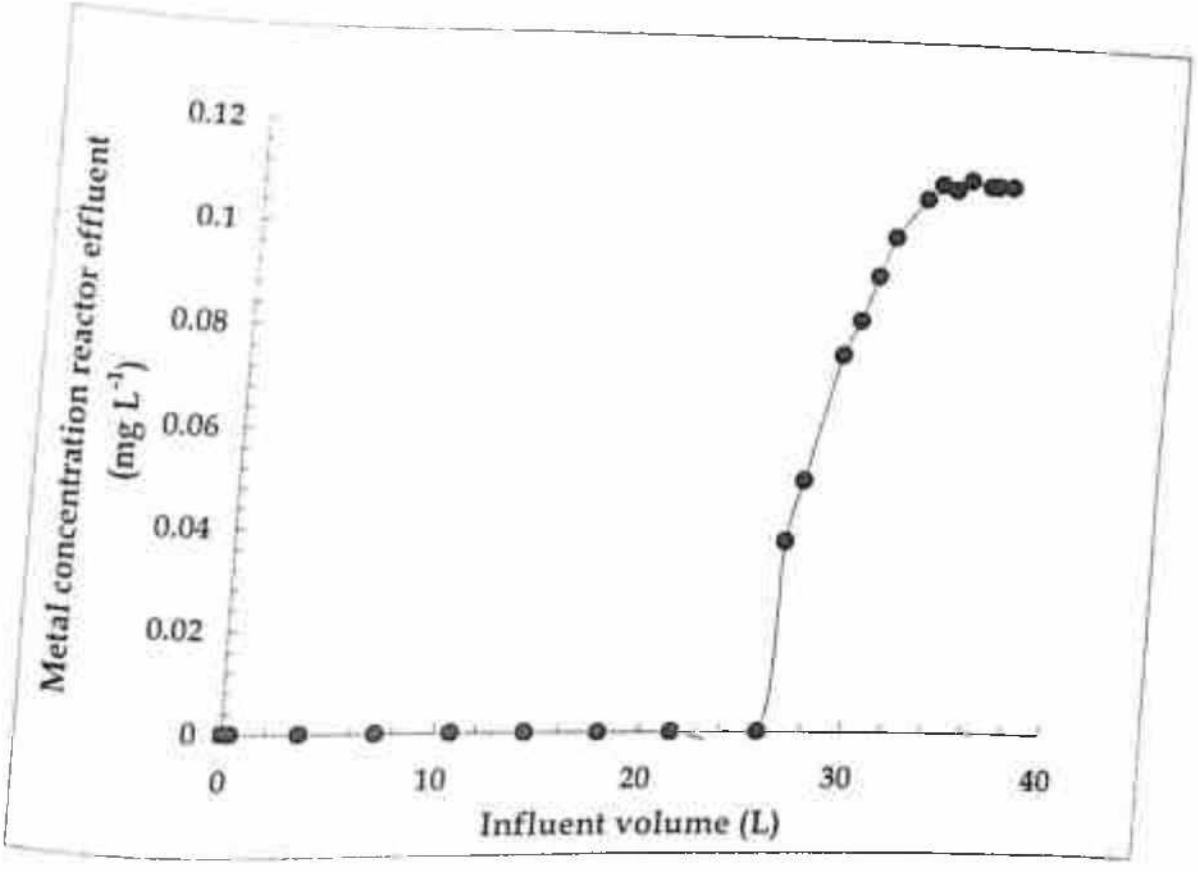

Fig. 33. Experimental breakthrough curve of Cu^{2+} sorption by PAG immobilized *S.platensis* biomass from simulated electro-plating industrial wastewater using packed bed column; The pH of influent 5.9 ± 0.1; Flow rate 6 mL min^{-1}.

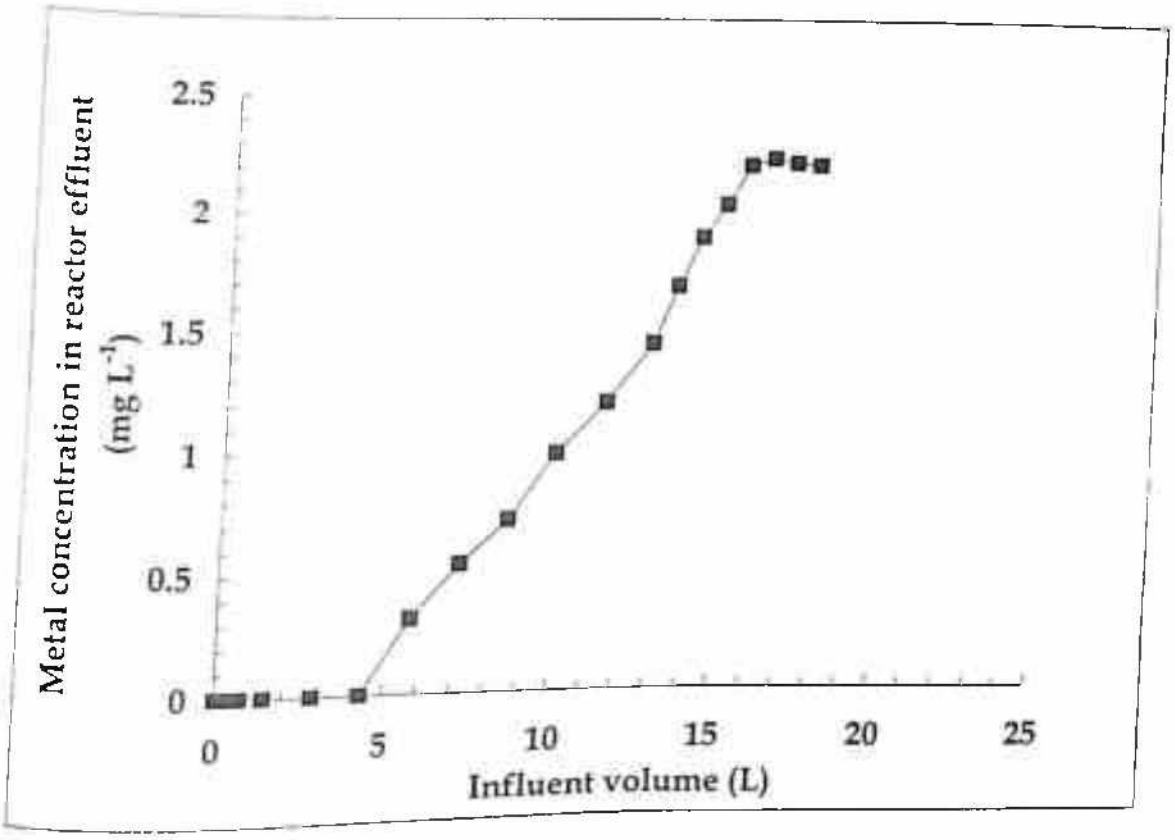

Fig. 34. Experimental breakthrough of Zn^{2+} sorption by PAG immobilized *S.plateusis* biomass from simulated electro-plating industrial wastewater using packed bed column; Flow rate 6 mL min^{-1}; The pH of influent 5.9 ± 0.1.

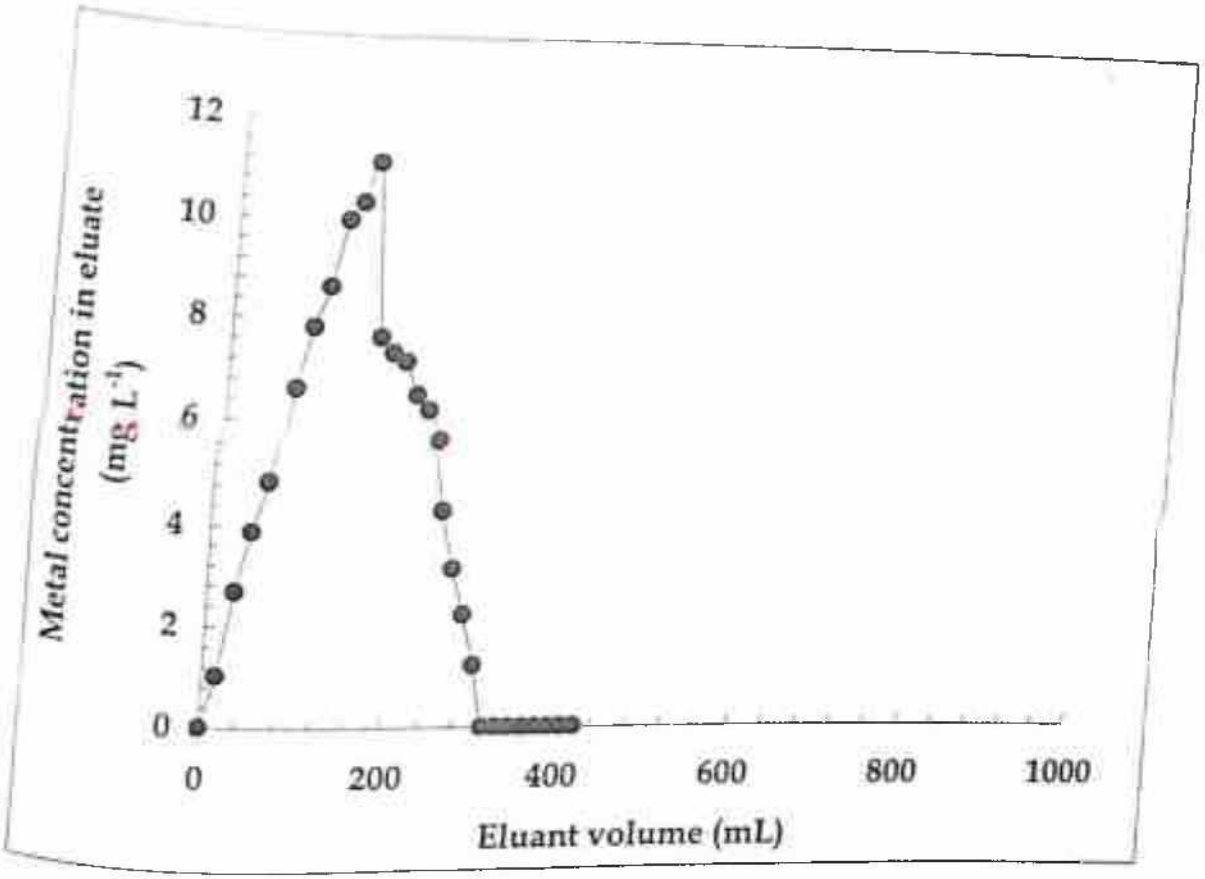

Fig. 35. Elution of Cu^{2+} from PAG immobilized *S.platensis* biomass in packed bed column; Flow rate 6 mL min^{-1}; The pH of eluant 5.8± 0.03.

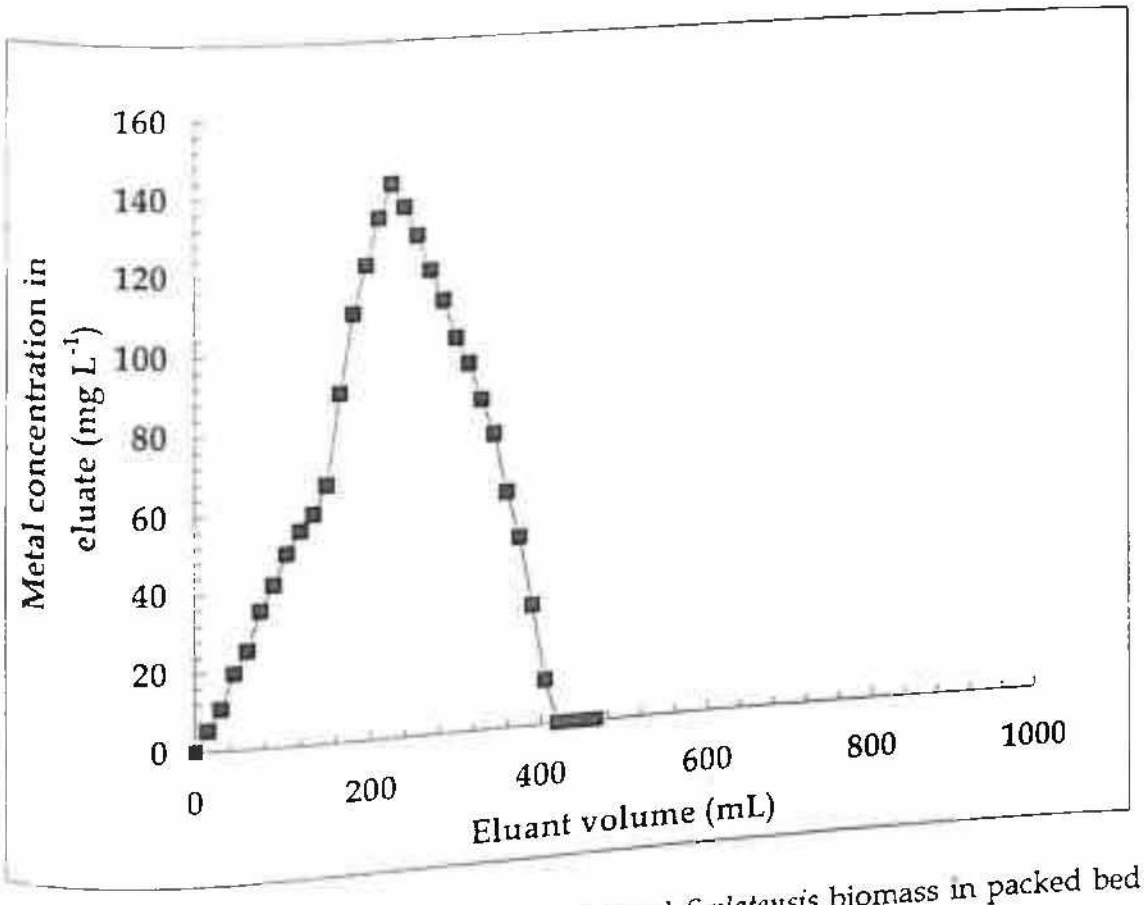

Fig. 36. Elution of Zn^{2+} from PAG immobilized *S.platensis* biomass in packed bed column; Flow rate 6 mL min^{-1}; The pH of eluant 5.7 ± 0.03.

Table-13. Performance efficiency of the column packed with PAG immobilized *S.platensis* biomass for removal and recovery of Cu^{2+} and Zn^{2+} from the simulated electro-plating industrial wastewater.

Metal	*Volume reduction (fold)*	*Concentration factor (fold)*
Cu^{2+}	86.3	101.1
Zn^{2+}	13.6	64.7

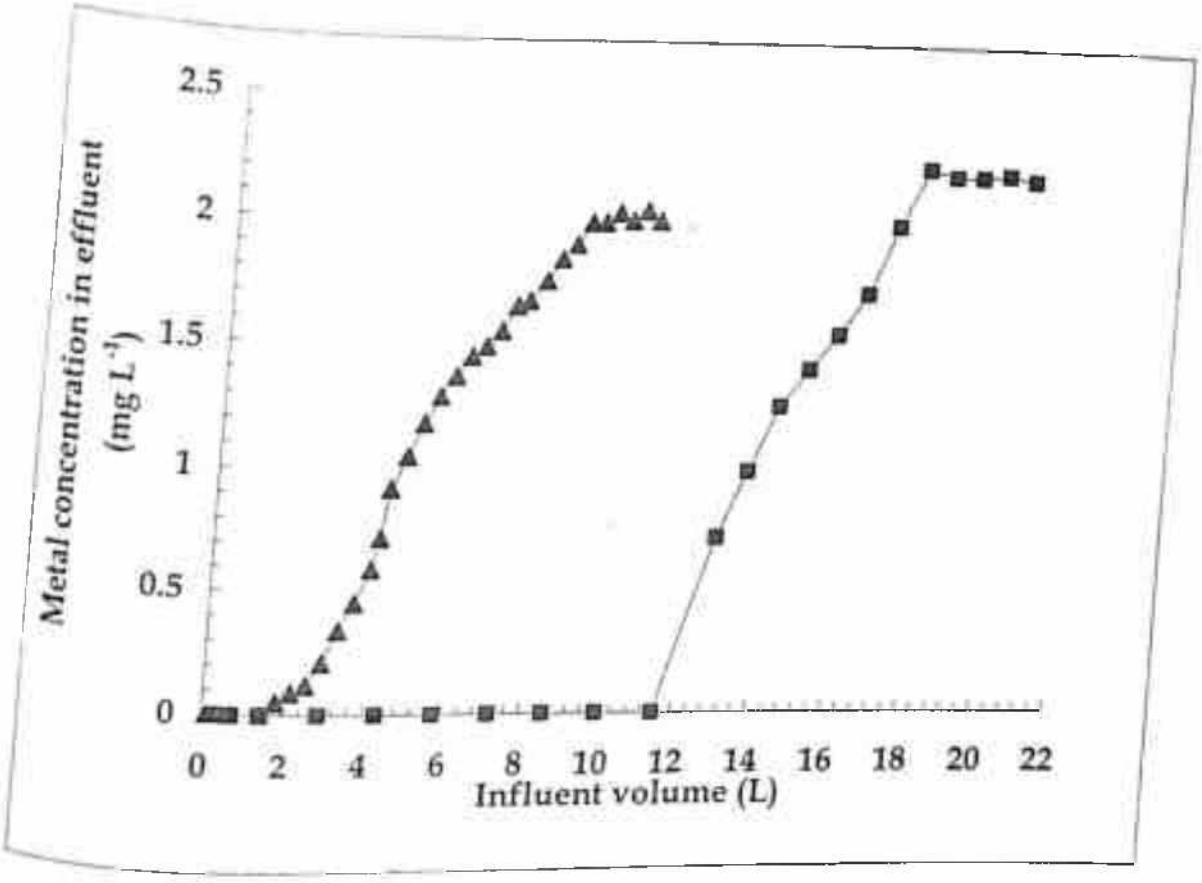

Fig. 37. Experimental breakthrough curve of ^{65}Zn and $^{85+89}Sr$ by PAG immobilized *S.platensis* using packed bed columnar reactor; The pH of influent 5.9 ± 0.1; Flow rate 6 mL min^{-1}; ^{65}Zn (■), $^{85+89}Sr$ (▲).

Discussion

Chapter IV
DISCUSSION

The removal of toxicants from wastewaters by biosorption has now become an established technique in bioremediation of industrial effluents (Wood and Wang 1984, White and Gadd 1990, Davis *et al.* 2000). The selection of biosorptive material (organism) is therefore crucial in a biosorbent-based removal of toxicants especially toxic metals. The selection of the requisite organismal biomass depends upon its metal sensitivity, metal accumulation potentials, stability under harsh environmental conditions and simpler nutritional requirement (Nakajima *et al.* 1981, Jeffers *et al.* 1991). In the present study, the cyanobacteria was chosen as a suitable group for their autotrophic nature and ability to adjust in changing environment over the other group of microbes (Fogg 1982, Borbely *et al.* 1990). The various strains tested for their tolerance towards different metals in the present investigation suggested that no single cyanobacterial strain showed supremacy in terms of tolerance of all metal ions examined. The toxic concentrations of tested metals varied with the cyanobacterial strain and the type of metal ions used (Table-3). The similar results have been reported earlier for Cu^{2+} toxicity in *Anabaena* sp. (Laube *et al.* 1980), Cd^{2+} toxicity in *Nostoc muscorum* (Singh and Pandey 1981), Ni^{2+} toxicity in *N. muscorum* (Rai *et al.* 1990) and Zn^{2+} toxicity in *Scenedesmus quadricauda* (Starodub *et al.* 1987). The metal toxicity in such cases is attributed to metal induced inhibition of photosynthesis (Conway 1978, Stratton *et al.* 1979), cell division (Singh and Pandey 1981), interaction with nucleic acid (Vallee and Ulmer 1972), competition for same enzyme binding site (Cataldo *et al.* 1983), blockade of sulfydryl group (Bonaly *et al.* 1980), N_2-fixation (Rai and Raizada 1986),

photosynthetic O_2 evolution (Verma and Singh 1991) amongst the several vital processes.

In order to select the suitable biomass for biosorption studies the various cyanobacterial strains were examined for their metal accumulating ability (Table-4). The algal biomass especially the cyanobacterial cells are known to accumulate high amounts of Cu^{2+} and other metal ions (Harris and Ramelow 1990, Mehta and Gaur 2001, Raveender *et al.* 2002). The pattern of metal accumulation, observed in the present study is more or less similar to the earlier report (Nakajima *et al.* 1981). A comparison of metal sensitivity and the maximum metal accumulation by different cyanobacterial strains showed *S.platensis* did not show maximum tolerance towards some metals but was superior in terms of metal accumulation. A strong positive correlation between the accumulation and the toxicity of Cu^{2+} as reported earlier by Singh and Verma (1988). No such correlation was found in the present investigation. Therefore, *S. platensis* with moderate metal sensitivity and highest metal accumulation, was selected amongst other cyanobacterial strains for further studies on biosorption.

The pH of the environment has been reported to be a determinant for specific and non-specific uptake of metal ions involving electron-rich functional groups located on the biomass (Zhou 1999, Sar and D'Souza 2001) as the hydrogen ion concentration is known to regulate metal toxicity and uptake in microbes (Babich and Stotzky 1982) and in algae (Whitton 1970, Singh and Yadava 1985). The present study on the effect of pH on uptake of Co^{2+}, Cu^{2+} and Zn^{2+} by *S.platensis* biomass indicates the inability of the cells to accumulate the metal ions at pH 3.0 or below (Fig. 4). This value, generally described as zero point or isoelectric point, is in agreement with the earlier reports on algal biomass (Harris and Ramelow 1990). The poor metal uptake at low pH (3.0) and increase in

uptake with increasing pH from 3.0-6.0 (Fig.4) is in agreement with the earlier report on U uptake in *P.aeruginosa* CSU strain where the pH profile of uptake followed a similar increase in metal loading with increasing pH (Hu *et al.* 1996). In general pH-dependent metal uptake could be the result of ionic attraction between metal ion and the cell surface as observed by Chang *et al.* (1997) for Pb, Cu and Cd biosorption by *Pseudomonas aeruginosa* PU21 and enhanced negative charge on the cell surface as described by Al-Asheh and Duvnjak (1995) for Cu^{2+} sorption by *Aspergillus carbonarius* biomass. It has been reported that under low pH conditions the cell surface becomes positively charged leading to competition between metal ions and hydrogen (H^+) as well as hydronium (H_3O^+) ions, reducing the affinity between biomass and metal ions (Gadd 1988, Hu *et al.* 1996 and Chang *et al.* 1997). The increased H^+ concentration suppressing metal uptake has also been observed in *Penicillium* (Galun *et al.* 1987) and *Rhizopus arrhizus* and *Streptomyces levoris* biomass (Treen Sears *et al.* 1984, Byerley *et al.* 1987). In contrast, higher pH (pH 5-6) resulted in increased metal uptake followed by a reduction. The uptake edge (the greatest change of uptake with in a narrow pH range) was found between 5.5 and 6.0. At pH above 6.0, the uptake was lower probably a result of decreased solubility of metals. Thus, the experiments on metal uptake were conducted at pH 6.0 using an acetate buffer (0.05 M) in the uptake mixture. The advantage of an acetate buffer system is that it enhances the metal solubility (Harris and Ramelow 1990). Since the biomass density is directly proportionate to the available metal binding sites (Harris and Ramelow 1990, Fourest and Roux 1992, Puranik and Paknikar 1999), it becomes an important parameter in the biosorption studies. Although the higher depletion of the metal from the medium was observed with increasing biomass density, the metal uptake for unit biomass showed a decline (Fig. 5). A possible reason for this

phenomenon is the lesser availability of surface area at higher biomass density and the rapid adsorption on to the microbial cell wall resulting into the lowered metal concentration in the solution. In case of low biomass density there is a high concentration gradient between the solutions and the microbial cell interior, which may be responsible for increased metal sorption. The present findings are supported by earlier report for metal sorption by *Phormidium laminosum* by Sampedro *et al.* (1995). This is attributed to reasons like limited availability of solute molecules (Fourest and Roux 1992), interference between binding sites at higher concentrations (deRome and Gadd 1987), electrostatic interactions and the reduced mixing at high biomass density (Meikle *et al.* 1990).

The concentration of metal ions in the ambient medium and the contact period is known to play an important role in metal uptake rates as well as in total accumulation of metal by the biomass (Verma and Singh 1990, Al-Asheh and Duvnjak 1995, Chang *et al.* 1997, Zhou 1999). The kinetics involved in metal uptake by *S.platensis* biomass indicates the bi-phasic metal uptake phenomenon with an initial rapid phase followed by secondary slower phase (Fig. 7). The initial phase was completed within 1 min of contact period contributing to 63 - 65% of the total metal uptake, where as the secondary phase continued up to 2 hr followed by biomass saturation. These results confirm the earlier finding of our laboratory on *Nostoc calcicola* (Verma and Singh 1990, 1991) in which the uptake of Cu^{2+} was characterized by a rapid metabolism-independent phase followed by a slower metabolism-dependent phase. Similar biphasic metal uptake has also been reported earlier by other investigators (Shehata and Whitton 1982, Les and Walker 1984, Peterson *et al.* 1984, Singh 1985, Campbell and Smith 1986) using a variety of cyanobacterial species and metal ions. The electrical attraction, chemical attraction or

Vander Wall's forces between the negatively charged cell surface ligands and metal cations have been suggested as the possible mechanism for rapid adsorption of metal (Weber 1972). The polar head group (Beveridge and Koval 1981) or the polygalacturonic acid like components of gram-negative bacterial outer membrane (Jellinek and Sangal 1972) play an important role in this process. The characteristic rapid phase of metal uptake has been suggested to precede the cellular assimilation of essential ions (Klatwasser and Frings 1980). The fast metal uptake shown by the organism shows the indication for its possible use in the construction of efficient biosorbent for further scale-up in continuous removal and recovery of heavy metals from industrial wastewater.

Since we attribute Cu^{2+} uptake by the *S. platensis* cells to a metabolism-dependent carrier mediated process, it was imperative to study the involvement of photosynthetic light and ambient temperature on the process. In contrast to the light independent Al^{3+} uptake in *Anabeana cylindrica* (Petterson *et al.* 1986), the observed partial reduction of Co^{2+}, Cu^{2+} and Zn^{2+} under dark conditions (Fig. 8) is in agreement with the earlier reports on dark-incubated cells of *N.calcicola* (Verma and Singh 1990) and Ni^{2+} uptake in *A.cylindrica* (Campbell and Smith 1986). A 76% uptake in dark cells could be attributed to the residual energy in dark-incubated cells, which possibly drives the first rapid phase of Cu^{2+} uptake process exhibited by the *S.platensis.*

The energy dependent processes are invariably temperature sensitive. The same is true for the cellular uptake of Co^{2+}, Cu^{2+} and Zn^{2+} under varying temperature conditions there was significant inhibition of metal uptake at temperature above 40°C or below 10°C (Fig. 9). This phenomenon may be attributed to the inactivation and denaturation of membrane proteins involved in the metal uptake process.

The metallic ions are always present in combination with other ions either in the natural ore or in the industrial, agricultural, domestic wastewaters. The results involving various combinations of Co^{2+}, Cu^{2+} and Zn^{2+} along with Cd^{2+} and Ni^{2+} on the uptake of target metals showed that the equimolar concentration had antagonistic effect in almost all the interactions examined (Table-5). The interaction amongst the heavy metals has shown to be synergistic, additive or antagonistic with respect to Cu^{2+} (Nielson and Nielson 1970, Hutchinson and Stokes 1975, Agarwal and Kumar 1977, Stratton and Corke 1979). The strong inhibition of Co^{2+} and/or Zn^{2+} uptake by Cu^{2+} than the other combinations indicates a higher affinity of *S.platensis* cells towards Cu^{2+} than the other ions examined. Such antagonistic effect could be related to the competition for the same binding site as reported by Cataldo *et al.* (1983) and Verma and Singh (1995). Another explanation for the selective uptake of these metal ions could be the differential stability constants leading to the characteristic hydration envelope for the individual metal ions (Andrea and Benno 1995).

Although several metals are required as micronutrient for the growth and metabolism, microorganisms show cation uptake often at concentrations high enough to be detrimental to them (Gadd and Griffiths 1978). Thus microbes have developed mechanisms that restrict intracellular metals in selected cell sectors and/or convert them to a more innocuous form such as insoluble phosphide, sulphide, carbide or hydroxide deposits (Gadd 1990, Ehrlich 1997, Davis-Hoover *et al.* 1998). In an effort to locate the sequestered Cu^{2+}, thin sections of Cu^{2+} equilibrated living cells of *S. platensis* biomass were examined by scanning and transmission electron microscopy. As shown by the electron micrographs (Plate 4-5), the cell walls of cyanobacterial cells were the main site for the Cu^{2+} deposition. Such localized cation deposition on the gram-negative cell

envelope has been attributed to the anionic character of cell wall and membrane components (Beveridge 1981, McLean *et al.* 1996). These observations on Cu^{2+} deposition throughout the cell boundary is also similar to the earlier study, showing preferential sequestration of intracellular lanthanum, silver or cadmium in and around the cell periphery, and leaving a small fraction for the cytoplasm in *P.aeruginosa* (Mullen *et al.* 1989, Wang *et al.* 1997). Kuyucak and Volesky (1989c) also reported similar observations with marine algae *A.nodosum* for Co^{2+} biosorption involving carboxyl, sulfydryl, phosphate, hydroxyl, amino and amide present in proteins and polysaccharides present in the cell membrane.

Elucidation of metal uptake mechanisms may provide a useful basis for manipulation and improvement of the biosorbents selectivity for the desired metal. Increased selectivity of biosorbent materials would undoubtedly result in higher efficiency of the metal concentration process, which in turn would positively affect the economics of the overall process. Despite the several peaks observed in IR spectrum, some characteristic peaks can be assigned to the native biomass prior to and after metal treatment (Fig. 10). The native biomass comprising different functional groups, which regulate the possible cell-cation interactions like H-bonding, complexation, etc. selectively gives following information on accumulation Cu^{2+}. In native biomass the amino group presents characteristic absorption at 3500-3000 cm^{-1} (N-H stretching) and at 1200-900 cm^{-1} (C-N stretching). The N-H stretching peak lays in a spectrum region occupied by a strong bond (3600-3000 cm^{-1}) that is due to presence of hydroxyl groups involved in hydrogen-bonding to various degrees. The IR analysis was made without using a drier box therefore the presence of intense OH peaks in the spectrum could be due to the great hydroscopicity of biomass and the presence of hydroxylic groups in the lyophilyzed

biomass. The sharp peak at 3000-2800 cm^{-1} is attributed to the C-H stretching that is characteristic of biological samples. For the native biomass other sharp peaks at 1650 cm^{-1} and 1550 cm^{-1} are attribute to the presence of amide-I and amide-II bands, respectively (for cellular proteins), followed by carboxylic bands at 1600 cm^{-1} and 1400 cm^{-1}. The absorption under 1300 cm^{-1} are characteristic peaks of phospate - carrying components, oligo - and polysaccharides of the cell wall. The weak absorption peaks 720 cm^{-1} and 790 cm^{-1} may be attributed to the glycoside bonds in the polysaccharide structure of the biomass. These findings are in conformity with earlier studies on cobalt sorption by an alga (Kuyucak and Volesky 1989c) and Cu^{2+} uptake by *Azospirillum brasilense* (Kannev *et al.* 1997). The IR spectrum of Cu^{2+} treated cells indicated no shifts or change in any of the characteristic absorbance bands exhibited by the native biomass. The only discernible changes observed were in the length, width and intensities of the peaks. Especially, there was 40-45%reduction in peak lengths that are attributed to amide and amino bands. A major change in sharpness and intensity of the peak was found, which may be attributed to carboxylic band. There was minor change in peak intensity of N-H stretching related to amino band. The changes in above-mentioned IR spectrum of metal treated cells indicate the possible involvement of amide, amino and carboxyl groups in Cu^{2+} accumulation. These changes in peak intensities must be interpreted as changes in concentrations rather than structural changes as described by Kuyucak and Volesky (1989c) for cobalt biosorption on to *A. nodosum*. The envelope of gram-negative bacteria consists of two membrane bilayers that sandwich a thin peptidoglycan layer between them. The outer membrane, characterized by a lipopolysaccharide (LPS) and phospholipids, is composed essentially of phosphate moieties that are able to bind metallic cations. Carboxyl moieties present, for example, in

the peptidoglycan and the LPS also constitute sites of high affinity for metallic cations (Texier *et al.* 2000). According to Kuyucak and Volesky (1989c), these observations might be an indication of involvement of ion-exchange phenomenon. In addition, the rapid kinetics of the reversible sorption-desorption cycle and the fact that the metal uptake process relatively insensitive to temperature, further substantiate the plausibility of the ion-exchange process being the prevailing mechanism in the sequestration of Cu^{2+} by *S. platensis* biomass. This clearly indicates the present interaction as a reversible physico-chemical process with a considerable potential for technological process exploitation.

Microbial biomass in its native form is not suitable for large-scale process utilization because of its particle size, low mechanical strength, and difficulty in separating them from the liquid stream due to relatively low density (Tsezos 1988). Use of immobilized cell systems is considered as a better approach for incorporating bacterial biomass in to an engineering process. Physical entrapment of organisms inside a polymeric matrix is one of the most widely used techniques for whole-cell immobilization (Scott 1987). The appropriate loading of biomass into matrix affects its stability leading to the sufficient availability of porus channeling, which influence metal ion transport to the core of the matrix (Bradenberger and Widmer 1999). In the present investigation, identification of a suitable immobilization matrix for *S.platensis* biomass was carried out using two matrices namely 1) Calcium alginate (Ca-Alg) and 2) polyacrylamide gel (PAG). A comparison of mechanical strength and metal uptake by the biomass (normal or lyophilized) loaded into these two gels clearly indicate superiority of the polyacrylamide gel immobilization over calcium alginate immobilization (compare Fig.17 with Fig.18). These observations are in conformity with the results obtained by Hu and Reeves (1997)

on U sorption by immobilized *P.aeruginosa*. They have recommended the use of covalently linked materials such as polyacrylamide gels over ionically cross-linked polymers such as calcium alginate gels, as the latter are not chemically stable due to the presence of anionic groups in effluents. The higher metal uptake in the range of ca. 3-5% by the empty (without biomass) calcium alginate beads has been attributed to the higher metal affinity of polyguluronic and polymuramic acid and hydroxyl groups present in alginate (Rees and Welsh 1997). On the other hand negligible metal uptake by empty PAG cubes may be due to presence of amino groups in the gel (Pons and Fuste 1993, Wong *et al.* 1993, Hu and Reevs 1997, Tucker *et al.* 1998). A comparison of metal uptake by the PAG cubes containing different biomass concentration established that a 28% (w/w) loading of biomass in PAG cubes was optimal for mechanical strength and metal uptake process. This optimal values of biomass loading is almost similar to the *P. aeruginosa* strain CSU immobilzed in polyurethane hydrogel (PUG) matrix (30%) observed by Hu and Reeves (1997). Hence, PAG cubes were used as the immobilization matrix in the present investigation due to their optimal metal uptake and appropriate mechanical strength. The experiments were conducted with ca. 28% of PAG immobilized *S. platensis* biomass. The *S.platensis* biomass showed highest affinity for Cu^{2+} followed by Zn^{2+} and Co^{2+} under immobilized condition similar to the free cells. Even PAG immobilized lyophilized biomass retained same efficiency of Co^{2+}, Cu^{2+} or Zn^{2+} uptake indicating that the PAG immobilization process does not inactivate or interfere with the metal binding sites of the biomass as observed by other workers for polyurethane hydrogel immobilization (Hu and Reeves 1997).

Equilibrium sorption studies are useful as they provide the vital information regarding the maximum sorption (loading capacity) value characterizing the sorbent performance

at high residual metal concentration, and the trend of the biosorption isotherm (Volesky 1988). Sorption of Co^{2+}, Cu^{2+} and Zn^{2+} by lyophilized, oven-dried and PAG immobilized biomass of *S.platensis* was studied by using the biosorption equilibrium isotherms (Fig. 13 and 20). The biosorptive capacity of *S.platensis* for the three metals investigated in the present study showed more or less hyperbolic kinetics with increasing metal concentrations. The lyophilized biomass in free and immobilized form showed better performance over oven-dried biomass as it showed higher metal loading at low equilibrium concentrations. The steep nature of Cu^{2+} isotherm and higher Cu^{2+} loading capacity ($q = 44$ mg g^{-1}) of free lyophilized biomass at low equilibrium concentration ($Ce = 9.9$ mg L^{-1}) indicates that the sorbent used would be efficient even at low residual concentrations. The same pattern of higher metal loading at low equilibrium concentration was noticed with immobilized form as well. A similar trend has been reported for cobalt sorption by *Ascophyllum nodosum* (Kuyucak and Volesky 1989a) as well as for the Th sorption by *Rhizopus arrihizus* and *Penicillium chrysorgenum* (Tsezos and Volesky 1981). The overall observations indicate that the lyophilized *S platensis* biomass is a potent sorbent of Co^{2+}, Cu^{2+} and Zn^{2+} with a preference for Cu^{2+} followed by Zn^{2+} and Co^{2+}. More than 15% metal loading capacity of biomass (w/w) has been defined as an economic threshold for practical applications of biosorption when compared with alternative methods such as traditional adsorption, ion-exchange, chemical precipitation, solvent extraction and reverse osmosis (Hu *et al.* 1996). The linearized metal sorption isotherms for all the metals were further analyzed for closeness of fit of langmuir and freundlich models (Fig. 14 and 21). These studies clearly indicate that the available experimental data fit reasonably well to both the models at all

concentrations with a preference to former as its correlation coefficient (r^2) values were highest than the latter (Table 6 and 10).

The efficient biosorption process also includes the recovery of bound metal from the loaded biomass as its integral process parameter. From the process point of view, elution of bound metal and regenerability of biomass enables the reuse of the biomass in multiple uptake and elution cycles allowing for greater process economy (Volesky 1987). Further the weak nature of metal-binding forces makes it possible to wash the bound metal from the biomass in higher concentrations. Our early experiments on effect of photosynthetic light and effect of temperature on metal uptake showed that a major part of uptake process, is a passive, metabolic energy independent phenomenon. Noticeably, more than 92% of Co^{2+}, Cu^{2+} or Zn^{2+} could be effectively recovered from the lyophilized and oven-dried biomass by few Ca-salts, Na_2-EDTA and mineral acids (Table-7 and 8). In the case of PAG immobilized *S. platensis* biomass, the metal recovery was found to be ca. 91% of total bound metal by same reagents (Table-11). In general, mineral acids showed higher elution of metal followed by Ca-salts or Na_2-EDTA depending on the metal examined. However, mineral acids were not used in further studies because of their damaging effects on biomass as also reported earlier in case of *R. arrhizus* biomass (Tsezos 1984). There was no apparent deleterious effects when Ca-salts or EDTA were used in study. Further Ca-salts were preferred over EDTA salt as EDTA readily forms complexes with metal ions, which tend to migrate much easier than the metals. Hu and Reeves (1997) reported that 90% desorption of U from *P. aeruginosa* strain CSU immobilized in Polyurethane hydrogel by 0.01M sodium carbonate. Ca, a constituent of plant cells, is abundant in nature and competes for electrostatic non-specific sites harbouring metal ions; 2000 mg L^{-1}. Ca can displace the total exchangeable cations in

soil by mass exchange (Pickering 1979). In the present investigation, while 10 mM $CaCl_2$ was found to elute 91–95% of the bound Co^{2+} or Zn^{2+} from lyophilized, oven-dried or PAG immobilized *S. platensis* biomass and 10 mM $Ca(NO_3)_2$ was noticed to elute 92–95% of the bound Cu^{2+} from the metal bound biomass. The selective preference of an eluant by the bound metal can be explained by the stability constants derived from the metal ligand formation (Andrea and Benno 1995). The selective metal elution has been suggested to result from competition of metal ions and electrostatic non-specific interactions with biomass (Flemming *et al.* 1990). The high eluting efficiency showed by Ca-salts in the present investigation is in agreement with earlier reports on Cd^{2+} and Cu^{2+} elution from marine algae (Davis *et al.* 2000) and on Th elution from *Pseudomonas* biomass (Sar and D'Souza 2002). Asthana *et al.* (1995) found $Ca(NO_3)_2$ to be effective in remobilizing Ni^{2+} (84%) from metal loaded biomass in comparison with NaCl. An added advantage is that $CaCl_2$ and $Ca(NO_3)_2$ are very cheap and are mild to the biomass. This makes large-scale operations cost-effective with non-destructive elution of Co^{2+}, Cu^{2+} or Zn^{2+} by the *S.platensis* biomass. The metal elution was found to be monophasic phenomenon involving swift metal stripping from the loaded biomass within 5–10 min of contact.

The studies conducted on *S.platensis* free lyophilized or oven-dried biomass in bimetallic combinations at equimolar concentrations of different metals showed anatagonistic effect in all interactions examined (Table-9). Among all metals examined, the Cu^{2+} was found to be potent inhibitor of Co^{2+} or Zn^{2+} uptake. Similarly Zn^{2+} showed more antagonism on Cu^{2+} uptake than Co^{2+} did for Cu^{2+}. The inhibition in uptake offered by Ni^{2+} was more towards Co^{2+} followed by Zn^{2+} and Cu^{2+}. Similarly the inhibition shown by Cd^{2+} was least among the metals studied. Even at the highest inhibition offered by

other co-ions examined, the present biomass could retain its ca. 45% of Co^{2+}, 91.5% of Cu^{2+} and 84% Zn^{2+} uptake capacity in bimetallic combinations. The highest selective uptake of Cu^{2+} in presence of other metals suggests that *S.platensis* prefers to accumulate Cu^{2+} over other metals tested. Similar observation was recently reported on *C.vulgaris* for Cu^{2+} sorption in presence of Ni^{2+} at equimolar concentration (Mehta and Gaur 2001). The selective preference of metals for the biomass binding sites can be explained by the differences in their stability constants that lead to metal binding on to the surface ligands apart from the characteristic hydration envelope for the individual metal ions (Andrea and Benno 1995). To better understand the co-ion effect on target metal ion, a detailed study was conducted with PAG immobilized *S.platensis* biomass. The effect of co-ion on the sorption of the target metal ion was examined under increasing co-ion concentration with fixed concentration of target ion. The target metal uptake was found to decrease with the increase in co-ion concentration. Similar reports of reduced uptake of Cd^{2+} by *A.nodosum* with increasing concentration of Zn^{2+} or Cu^{2+} (Volesky and Holan 1995) also support our observation and the observation of DeCarvalho *et al.* (1995), Lee and Volesky (1999) on Cu^{2+} sorption by an algal biomass.

The biomass reversibility over multiple cycles of metal sorption and elution is an essential key factor that determines scale-up of metal recovery technology. The results obtained from the batch experiments conducted with PAG immobilized *S.platensis* biomass clearly indicate the suitability of biomass for at least 7 continuous cycles of sorption and elution of metal with 100% efficiency (Fig. 23). Even at the tenth cycle, the biomass retained ca. 43% of its sorption and ca. 55% elution capacity. Similar results were noticed with *P. aeruginosa* strain CSU immobilized in PUG beads for continuous U biosorption-desorption (Hu and Reeves 1997) leading to full efficiency for 10 cycles

using 0.01 M sodium carbonate as desorbing agent. In an another report (Puranik and Paknikar 1999) for continuous Pb, Cd and Zn sorption-desorption by *Citrobacter* strain MCM B-181 immobilized in polysulfone matrix, investigators used 0.1M HCl and 0.1M EDTA as desorbing agent and concluded that 0.1M EDTA is an ideal one in order to avoid the deleterious effects of acid on biomass. In contrast to the decreased efficiency in sorption and elution in the present study, Wong *et al.* (1993) reported enhancement in Cd adsorption by *P.putida* II-11, which is attributed to the HCl induced structural changes as HCl was used as eluant. The enhanced Cd sorption by *P.aeruginosa* PU 21 treated with HCl also reported by Chang *et al.* (1997) over multiple cycles of sorption and elution but they could not find the similar trend with Cu and Pb which is attributed to the loss in cell concentration from the working solution after repeated centrifugation and rinse operations. Further, they recommended the use of immobilized cell systems in order to minimize the biomass loss due to solid-liquid separations. A comparison of sorption/elution of metals by free and immobilized *S.platensis* biomass showed similar values suggesting that immobilization of biomass has no effect on the efficiency of biomass, however, calcium alginate immobilization is known to increase Cu^{2+} sorption by *N.calcicola* (Singh *et al.* 1989).

For the continuous metal removal and recovery the fixed bed column, which optimizes the concentration difference is known to be the most effective process (Volesky and Schiewer 1999). In order to optimize the process, the efficiency of columnar reactor packed with 2 g of PAG immobilized *S.platensis* biomass was assessed in terms of metal removal and recovery under four different Residence Time (RT). As the residence time decreased (with the increase in flow rates) the amount of influent passed through the column also decreased leading to early breakthrough (Fig. 28 and 30). Similarly, the

amount of eluant requirement increased with the decrease in RT (Fig. 29 and 31). However, the highest value of volume reduction (104-fold) was obtained at the RT of 4.6 min in the present study (Table-12) is much higher than the 55-fold reduction reported by Fogarty *et al.* (1999) for Cu^{2+} using epichlorhydrin-immobilized *Azolla* biomass. The concentration factor, which is a widely used parameter for assessing the efficiency of any was found to be very high (109-fold) in the present study (Table-12). Such consideration becomes vital due to increasing costs of solid waste disposal, with selective removal of metals from the metal loaded biomass (Eccles 1995). The removal and recovery of Zn^{2+} also showed similar results with lower volume reduction and concentration factor (Table-12) as compared to Cu^{2+}, further establishing the higher affinity of the biomass towards Cu^{2+}.

Biosorbent reusability and selective recovery of metals over multiple runs are considered to be critical in the development of a viable, cost-effective metal bioremediation technology (Wilhelmi and Duncan 1995). This process should improve the process economics of a fixed bed biosorption system (Volesky 1990). For the efficient use of columnar reactor technology, recycling of PAG immobilized *S.platensis* biomass was tested in ten cycles of Cu^{2+}loading and elution at the optimal flow rate of 6 mL min^{-1} (as established earlier). For the first seven runs of metal loading and elution, the column showed ca. 100-fold volume reduction and 105-fold concentration factor (Fig 32a and b). These values are similar to the batch studies discussed earlier emphasizing the optimal performance of the columns for at least seven such cycles. A study conducted by Tsezos *et al.* (1989) reported equal efficiency of U sorption and elution over 12 such cycles with *R.arrhizus* biomass.

In an effort to examine the present biosorbent under real life industrial conditions, the column was challenged with simulated electro-plating industrial effluent containing Cu^{2+} and Zn^{2+} at the flow rate of 6 mL min^{-1}. The results show that the presence of one ion decreased the column performance for the other ion (Table-13). The Zn^{2+} inhibited 17.3% and 7.6% of Cu^{2+} volume reduction and concentration factor respectively. Similarly, the Cu^{2+} inhibited 64.4% of volume reduction and 22.4% concentration factor values. Conversely, the efforts made to examine the PAG immobilized *S.platensis* biomass to remove radionuclides from the aqueous solutions, the columnar reactor was challenged with ^{65}Zn and $^{85+89}Sr$ containing feed at the flow rate of 6 mL min^{-1}. The removal of ^{65}Zn was found to follow a similar pattern to the non-radioactive Zn^{2+} with no difference (compare Fig. 36 with Fig. 30), clearly indicating the suitability of the present technology towards the nuclear waste treatment. For some specific applications in nuclear waste treatment, the cheaply available biomass is not regenerated instead incinerated. On the other hand, the removal of $^{85+89}Sr$ was found to be lesser compared with other metals examined so far in the study suggesting the lower preference of biomass towards the $^{85+89}Sr$.

Chapter V
SUMMARY

The investigations were carried out aiming at identification of suitable biosorbent for removal and recovery of heavy metals including radionuclides, generated at the various point sources of industrial complexes. The study was conducted with respect to metal sensitivity, metal uptake under various environmental conditions, entrapment of biomass into gel matrix and its use in removal and recovery of heavy metals using up-flow packed bed columnar reactor. The results obtained hitherto are summarized as follows:

1. The toxicity studies involving Mn^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Cs^{2+} and Sr^{2+} with cyanobacterial strains indicated that *Spirulina platensis* was moderately tolerant towards the tested metals.
2. The Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+} and Sr^{2+} accumulation studies by various cyanobacterial strains showed the highest accumulation of these metals by *S.platensis* cells. Thus, detailed accumulation processes in characterization of Co^{2+}, Cu^{2+} and Zn^{2+} uptake were carried out using *S.platensis* under normal, lyophilized or oven-dried conditions.
3. The effect of pH on metal availability clearly indicated that Co^{2+}, Cu^{2+} or Zn^{2+} ions are not available in the solution pH above 6.0. The maximum metal uptake by *S.platensis* biomass was observed at pH 6.0 with free (normal, lyophilized and oven-dried) or immobilized biomass.

4. A cell density of 0.5 - 1.0 mg mL^{-1} of *S.platensis* biomass under normal, lyophilized and oven-dried conditions, was found to be optimal for metal uptake process.

5. The metal uptake process in *S.platensis* biomass under free (normal, lyophilized or oven-dried) or immobilized condition was found to be dependent on initial metal concentration. The maximum metal uptake (w/w) was observed with normal biomass of *S.platensis* (Co^{2+} 18.1%, Cu^{2+} 27% and Zn^{2+} 25%) followed by lyophilized (Co^{2+} 7.9%, Cu^{2+} 25.0% and Zn^{2+} 11.1%) and oven-dried (Co^{2+} 2.6%, Cu^{2+} 16.7% and Zn^{2+} 3.5%) biomass. The sorption isotherm was further analyzed by applying the langmuir and freundlich models. Though both the models could moderately represent uptake process, the data showed preference towards langmuir model.

6. The time-dependent metal uptake revealed the existence of two phases involving a rapid phase, which lasted for 1-2 min contributing to 65-75% of total uptake followed by a slower phase that continued for 2hr of metal exposure.

7. The Co^{2+}, Cu^{2+} or Zn^{2+} uptake process in normal *S.platensis* cells was found to be light - and temperature - dependent.

8. The metal elution studies conducted using various reagents showed more than 90% elution with mineral acids, calcium salts and Na_2EDTA with free (lyophilized and oven-dried) as well as immobilized biomass. Due to the deleterious effects on biomass and environmental contamination posed by mineral acids and Na_2EDTA, a 10 mM $Ca(NO_3)_2$ was selected for Cu^{2+} elution whereas 10 mM $CaCl_2$ was found to be most suitable for Co^{2+} and Zn^{2+} elution owing to their lower economics and milder nature towards biomass.

9. The presence of Cu^{2+} in the uptake solution showed maximum inhibition of Co^{2+}, and Zn^{2+} uptake process in comparison with other ions whereas least inhibition was found in case of Cd^{2+}.

10. The experiments conducted to immobilize the *S.platensis* biomass for possible use at larger scale clearly established superiority of polyacrylamide gel (PAG) as the immobilization matrix over Calcium alginate (Ca-Alg) owing to its higher mechanical strength and the ability to retain the biomass accumulation capacity. The lyophilized *S.platensis* cells were found to be better suited for entrapment than the normal cells. The studies conducted to examine the suitability of immobilized *S.platensis* biomass over multiple cycles of Co^{2+}, Cu^{2+} and Zn^{2+} sorption and elution showed that same PAG cube can be reused for at least seven cycles of sorption with full efficiency followed by 28-32% reduction in both the process in the subsequent cycles.

11. For continuous removal and recovery of Cu^{2+} or Zn^{2+} from the solution containing these ions alone or in combination at the ratio of 1:22 (as found in electro- plating industrial effluent), the up-flow fixed bed columnar reactor was optimized for Residence Time (RT) by altering the flow rate. At the RT of 4.6 min, the columnar reactor packed with PAG immobilized *S.platensis* showed highest values of volume reduction factor (VRF ca. 104-fold) and concentration factor (CF ca. 109-fold) for Cu^{2+} followed by Zn^{2+} (ca. 38-fold VRF and 83-fold CF). When the simulated electro-plating industrial effluent was studied under same RT, the VRF and CF for both Cu^{2+} and Zn^{2+} were inhibited and the reduction was very high for Zn^{2+} than for Cu^{2+}. The reuse of PAG immobilized *S.platensis* in columnar reactor for Cu^{2+} removal and recovery over multiple

cycles indicated the possibility of using the same biomass over seven consecutive cycles with 99% efficiency of sorption and elution using 10mM $Ca(NO_3)_2$ as eluant confirming the same observation found in batch studies. Overall seven cycles of metal removal and recovery was found to process ca. 210 L of influent before the breakthrough.

12. The experiments involving the removal of radioisotope ^{65}Zn by the similar up-flow reactors packed with PAG immobilized *S.platensis* biomass, showed that the system worked with similar efficiency as that of non-radioactive Zn^{2+} removal. However, the system did not work well for $^{85+89}Sr$ removal indicating the lower affinity of tested biomass towards Sr^{2+}.
13. The efforts made to elucidate the Cu^{2+} localization in *S.platensis* biomass using scanning and transmission electron microscopy techniques revealed that the cell exterior is the major metal binding site though there was minute entry of metal into cytoplasm. The infra-red spectrum of metal treated *S.platensis* biomass indicated the possible involvement of amide, amino and carboxyl groups in metal binding.

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