Universidade do Porto
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Removal and Selective Recovery of Heavy Metals from Electroplating Effluents

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A thesis submitted in partial fulfilment of the requirement for

the degree of

Doctor in Environmental Engineering

by
University of Porto

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requimte
rede de química e tecnologia

ISEP
INSTITUTO SUPERIOR
DE ENGENHARIA DO PORTO
POLITÉCNICO DO PORTO

Porto, August 2010

POPH
PROGRAMA OPERACIONAL POTENCIAL HUMANO

QREN
QUADRO DE REFERÊNCIA ESTRATÉGICO NACIONAL

UNIÃO EUROPEIA
Fundo Social Europeu

To

My mother, Maria

The memory of my father, Paulo

Acknowledgments

At the end of this thesis I would like to acknowledge all those who, of several ways, have made it possible.

First of all, I would like to express my deep gratitude to my supervisors, Professor Helena Soares and Professor Eduardo Soares, for giving me the opportunity to work on such an interesting project and for their great and continuous scientific guidance, advice, support and encouragment. It has been a pleasant and proficuous learning process throughout these last years.

For the facilities that allowed me to conduct the scientific experiments from which this thesis results, I would like to express my appreciation to the Superior Institute of Engineering from Porto Polytechnic Institute, Department of Chemical Engineering of Faculty of Engineering of Porto and REQUIMTE.

I am also grateful to "Fundação para a Ciência e a Tecnologia" for the grant financed under the Project POCTI/CTA/47875/2002 and for a PhD scholarship with reference SFRH/BD/31755/2006.

I wish to thank Mr. Emanuel Monteiro from "Instituto de Ciências Biomédicas Abel Salazar" for gently drying yeast cells by critical point for SEM examination and Engenheiro José Luís Moreira for technical assistance with FTIR.

Moreover, I am grateful to Professor Rui Boaventura for kindly facilitating the use of microwave digestor and ionic chromatography equipment.

I would also like to thank my colleagues from LAB 302B, namely Carina, Cristina, Gina and João. Thank you for the lovely working environment, your kind support and friendship.

Another essential contribution came from the amazing people that I have met during the last years: Adriano, Afonso, Albina, Alejandro, Ana, Filipa, Herney, Luisa, Miguel, Mónica, Nuno, Olga, Pedro Martins, Ratola, Rui, Susana, Vânia, ... Thank you all for the friendship and for the good times we shared.

A very special thanks to my closest family for their support, dedication, care and patience. To my mother, Maria, and to my father, Paulo (unfortunately you are not here anymore, but you remain alive in my heart), I mostly acknowledge the education, the unconditional love and the encouragment to follow my dreams. To my brothers, Isabel and António, I have to say that it is good to know that you will always be here for me and that your love and support will never cease. Finally, I wish to thank my nephew, Paulo, for always putting a smile on my face, even in the most difficult moments.

Resumo

As indústrias de galvanoplastia produzem grandes quantidades de efluentes com concentrações significativas de metais pesados. Estes efluentes, devido à toxicidade dos metais pesados, devem ser tratados antes de serem lançados no meio aquático ou enviados para estações municipais de tratamento de efluentes urbanos. As tecnologias físico-químicas convencionais para o tratamento destes efluentes apresentam-se como inadequadas ou dispendiosas. O problema da poluição dos metais tem promovido um interesse de investigação crescente no desenvolvimento de processos alternativos para a remoção e recuperação eficaz e económica de metais de efluentes industriais.

O objectivo desta tese consistiu em desenvolver um processo multi-etápico, à escala laboratorial, em que se combinam biossorção e tecnologias químicas, para remover e recuperar selectivamente metais pesados de efluentes de galvanoplastias.

Inicialmente foi investigada a aplicação de células da estirpe cervejeira *Saccharomyces cerevisiae* NCYC 1364, como biossorvente, na remoção de metais pesados usando a floculação como um processo de separação sólido-líquido. Com esse objectivo, a influência da concentração de biomassa, a presença de diferentes metais pesados (Cd^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+} e Cr^{3+}) e a temperatura de morte da biomassa no perfil de sedimentação da levedura foi estudada e optimizada. Posteriormente, foram realizadas cinéticas de sorção e estudos de equilíbrio para os iões cobre, níquel e zinco com células vivas e mortas por calor a 45 ºC. Adicionalmente, com o objectivo de elucidar as diferentes capacidades de acumulação da biomassa morta e viva, foram avaliadas as possíveis alterações estruturais e moleculares da biomassa morta. As células mortas mantiveram a sua capacidade floculante e apresentaram maior capacidade de acumulação de metais, sendo mais adequadas para a bioremediação de efluentes de galvanoplastia.

Mais tarde, foram realizados estudos de especiação química de forma a optimizar as condições experimentais a usar na bioremediação, nomeadamente a quantidade de biomassa e o número de ciclos necessários para remover eficientemente os metais pesados presentes em três efluentes reais provenientes de galvanoplastias. Estes estudos permitiram ainda prever o impacto de ligandos inorgânicos (carbonatos, cloretos, fluoretos, fosfatos, nitratos e sulfatos), que podem ser encontrados em efluentes reais, na eficiência da bioremediação dos metais.

Estudos experimentais confirmaram que os fluoretos podem reduzir a remoção de Cr^{3+}.

Um efluente real e efluentes sintéticos contendo um mono-elemento (Ni) foram tratados. A passagem por uma série de ciclos sequenciais contendo células de *S. cerevisiae* inactivadas por calor permitiu reduzir a concentração de Ni, após o segundo ou terceiro ciclo, a valores inferiores ao limite legal de descarga, de acordo com a Agência de Protecção Ambiental Americana e a legislação portuguesa, respectivamente. Isto corresponde a uma remoção de 89% de níquel. Adicionalmente, dois efluentes reais contendo multi-elementos (Cu, Ni e Zn) ou (Cr, Cu, Ni e Zn) foram tratados usando uma tecnologia híbrida que combina a precipitação química a pH 6.0 com um subsequente processo de base biotecnológica (usando células de leveduras mortas por calor). Após o terceiro ciclo de biomassa, as concentrações de metais baixaram para valores inferiores aos limites legais de descarga; remoções ≥ 89, 91, 92 e 94% foram obtidas para Ni, Cu, Cr e Zn, respectivamente. No caso de efluentes contendo Cr, duas estratégias foram consideradas: a redução de Cr^{6+} a Cr^{3+}, ajuste de pH a 6,0 e subsequente tratamento com uma série de ciclos de biomassa. Em alternativa, o Cr^{6+} foi selectivamente removido a pH 2,3 (98%) pela biomassa a pH 2,3; seguindo-se o ajuste de pH a 6,0 e o tratamento com uma série de ciclos de biomassa.

Finalmente, a biomassa contendo metais pesados foi incinerada e as cinzas digeridas com ácido num sistema assistido por microondas; os metais foram selectivamente recuperados, com elevado rendimento e pureza, combinando processos electroquímicos e químicos. O cobre foi recuperado, como cobre metálico, por electrólise a potencial controlado. O níquel e o zinco foram eficientemente recuperados, como hidróxidos metálicos, por precipitação alcalina sequencial e podem ser directamente reintroduzidos no processo produtivo ou mesmo comercializados. O crómio foi obtido, como uma solução de cromato, após oxidação de Cr^{3+}a Cr^{6+} em condições alcalinas. A solução de cromato pode ser reintroduzida nos banhos de crómio do processo produtivo das galvanoplastias.

Em conclusão, desenvolveu-se um ciclo praticamente fechado para a remoção e recuperação selectiva de crómio, cobre, níquel e zinco de efluentes de galvanoplastias.

Abstract

Electroplating industries produce large amounts of effluents containing significant concentrations of heavy metals. Due to heavy metals toxicity, these effluent streams should be treated before being discharged into the aquatic systems or sent to the municipal sewage treatment plants. Conventional physicochemical technologies for the treatment of these effluents appear to be inadequate or expensive. The problem of metal pollution has promoted a big research interest on the development of alternative processes for effective and low cost removal and recovery of metal-bearing industrial effluents.

The aim of this thesis was to develop a multi-stage process, at laboratorial scale, which combines biosorption and chemical technologies, to remove and selectively recovery heavy metals from electroplating effluents.

Firstly, the application of cells of the brewing strain of *Saccharomyces cerevisiae* NCYC 1364, as biosorbent, on heavy metal removal using flocculation as a solid-liquid separation process was investigated. For this purpose, the influence of biomass concentration, the presence of different heavy metals (Cd^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+} and Cr^{3+}) and the killing temperature of the biomass on the yeast settling profile was studied and optimized. Subsequently, sorption kinetic and equilibrium studies were performed for copper, nickel and zinc ions with live and heat-killed cells at 45 ºC. Furthermore, possible structural and molecular modifications of dead biomass were examined in order to elucidate the different accumulation ability of dead and live biomass. Dead cells retained flocculation ability and presented higher metal removal, being more suitable to be used in the bioremediation of electroplating effluents.

Later on, chemical speciation studies were carried out in order to optimize the experimental conditions to be used in the bioremediation, namely the amount of biomass and the number of batches required to remove efficiently heavy metals present in three real electroplating effluents. Chemical speciation studies also allowed predicting the impact of inorganic ligands (carbonates, chlorides, fluorides, phosphates, nitrates and sulphates), which can be found in real effluents, on metal bioremediation efficiency. Experimental studies confirmed that fluorides can reduce Cr^{3+} removal.

Real and synthetic effluents containing a mono-element (Ni) were treated. The passage through a series of sequential batches containing heat-inactivated cells of *S. cerevisiae* allowed reduce Ni concentration below legal limit of discharge, after the second or third batch according to the U.S.-Environmental Protection Agency and Portuguese law, respectively. This corresponded to a removal of nickel of 89%. In addition, two real effluents containing multielement (Cu, Ni and Zn) or (Cr, Cu, Ni and Zn) were treated using a hybrid technology, which combines chemical precipitation at pH 6.0 with a subsequent biotechnological-based process (using heat-killed yeast cells). After the third batch of biomass, metal concentrations were lowered to values below the legal limits of discharge; removals $\geq$ 89, 91, 92 and 94% were attained for Ni, Cu, Cr and Zn, respectively. In the case of effluents containing Cr, two strategies were considered: reduction of Cr^{6+} to Cr^{3+}, pH adjustment to 6.0 and subsequent treatment with a serial batch of biomass. Alternatively, Cr^{6+} was selectively removed (98%) by yeast biomass at pH 2.3; subsequently, pH of the effluent was raised up to 6.0 and treated with a serial batch of biomass.

Finally, heavy metal loaded biomass was incinerated and ashes were acid digested in a microwave assisted process; metals were selectively recovered, with high yield and purity, combining electrochemical and chemical processes. Copper was recovered, as metallic copper, by electrolysis at controlled potential. Nickel and zinc were efficiently recovered, as metal hydroxides by sequential alkaline precipitation, and can be directly reintroduced in the production process or being sold. Chromium was obtained, as a solution of chromate, after oxidation of Cr^{3+} to Cr^{6+} under alkaline conditions. Chromate solution can be reintroduced in the chromium baths of the electroplating production process.

In conclusion, a nearly closed cycle for removing and recovering selectively chromium, copper, nickel and zinc from electroplating wastewaters was developed.

Résumé

Les industries de galvanoplastie produisent de grandes quantités d'effluents contenant des concentrions significatifs de métaux lourds. Ces effluents doivent être traitées, étant donnée leur toxicité en métaux lourds, avant d'être lancées en milieu aquatique ou envoyées vers des stations municipales de traitements d'effluents urbains. Les technologies physique-chimies conventionnelles existantes pour le traitement de ces effluents sont inadaptées ou onéreuses. Le problème de la pollution des métaux est en train de produire un intérêt croissant de l'investigation dans le développement de procédés alternatifs pour retirer et récupérer efficacement et économiquement les métaux d'effluents industriels.

L'objectif de cette thèse fut le développement d'un processus en plusieurs étapes, à l'échelle de laboratoire, dans lequel s'assemblent biosorption et technologies chimiques, pour retirer et récupérer de forme sélective les métaux lourds d'effluents de galvanoplasties.

Initialement, l'application de cellules de la levure de la bière *Saccharomyces cerevisiae* NCYC 1364, comme biosorbant, pour retirer les métaux lourds utilisant la floculation comme processus de séparation solide-liquide, fut travaillée. Avec cet objectif, l'influence de la concentration de biomasse, la présence de différents métaux lourds (Cd^{2+}, Cu^{2+}, Ni^{2+}, Zn^{2+} et Cr^{3+}) et la température de mort de la biomasse dans le profil de sédimentation de la levure, fut étudiée et optimisée. Ultérieurement, des cinétiques de sorption et des études d'équilibre furent réalisés pour les ions cuivre, nickel et zinc avec des cellules vivantes et mortes à chaleur de 45ºC. En plus de cela, de possibles altérations structurelles et moléculaires de la biomasse morte furent évaluée ayant pour objectif d'élucider les différentes capacités d'accumulation de la biomasse morte et vivante. Les cellules mortes ont maintenu leur capacité floculante et ont présenté une plus grande capacité d'accumulation de métaux étant plus adaptées au bio-retirement d'effluents de galvanoplastie.

A postériori, des études de spéciation chimique furent réalisées de manière à optimiser les conditions expérimentales à utiliser dans la bioremédiation, telle que la quantité de biomasse et le nombre de cycles nécessaires pour retirer efficacement les métaux lourds présents dans trois effluent réels de galvanoplasties. Ces études ont permis en plus de prévoir l'impacte de ligands inorganiques (carbonates, chlorates, fluoroses, phosphorés, nitrates et sulfates), qui peuvent être trouvés dans des

effluents réels, dans la réussite de la bioremédiation des métaux. Des études expérimentales ont confirmées que les fluoroses peuvent réduire à enlever le Cr^{3+}.

Un effluent réelle et des effluent synthétiques contenant un mono-élément (Ni) furent traitées. Le passage par une série de cycles séquentiels contenant des cellules de *S. cerevisiae* inactivées par la chaleur a permis de réduire la concentration de Ni à des valeurs inférieures au limite légal de décharge, après le second ou troisième cycle en accord avec l'Agence de Protection de l'Environnement Américaine et la loi portugaise, respectivement. Cela correspond à retirer 89% de nickel. En plus, deux effluents réels contenant de multiples éléments (Cu, Ni e Zn) ou (Cr, Cu, Ni e Zn) furent traités par l'utilisation d'une nouvelle technologie hybride qui assemble la combinaison chimique pH 6.0 avec ensuite un procédé de base biotechnologique (utilisant des cellules de levures mortes par la chaleur). Après le troisième cycle de biomasse, les concentrations de métaux baissèrent à des valeurs inférieures aux limites légales de décharge, des transferts ≥ 89, 91, 92 et 94% furent obtenus pour Ni, Cu, Cr et Zn, respectivement. Dans le cas des effluent contenant Cr, deux stratégies furent considérés: la réduction de Cr^{6+} à Cr^{3+}, ajustement de pH à 6,0 et ensuite un traitement avec une série de cycles de biomasse. De forme alternative, le Cr^{6+} fut de façon sélective retiré à pH 2,3 (98%) par biomasse de levure à pH 2,3, se suivant l'ajustement de pH à 6,0 et le traitement avec une série de cycles de biomasse.

Finalement, la biomasse contenant des métaux lourds fut incinérée et les cendres digérées avec de l'acide dans un système assisté par micro-ondes, les métaux furent sélectivement récupérés, avec grand profit et grande pureté, combinant des processus électrochimiques et chimiques. Le cuivre fut récupéré, comme le cuivre métallique, par électrolyses à potentiel contrôlé. Le nickel et le zinc furent efficacement récupérés, comme hydroxydes métalliques par précipitation alcaline séquentielle et peuvent être directement réintroduits dans le processus productif ou même commercialisés. Le chrome fut obtenu, comme une solution de chromé, après oxydation de Cr^{3+} à Cr^{6+} dans des conditions alcalines. La solution de chromé peut être réintroduite dans les bains de chrome du processus productif des galvanoplasties.

Pour conclure, un cycle pratiquement fermé fut développé par retirement et récupération sélective du chrome, cuivre, nickel et zinc d'effluent de galvanoplasties.

Preface

Electroplating industries produce large amounts of effluents containing harmful metals that must be treated before being discharged into the aquatic systems. Conventional technologies, like precipitation and ion exchange, are not efficient on metal removal, economically prohibitive or even impracticable for the treatment of large volumes of effluents containing low metal concentrations. Additionally, these processes are based on phase transference of metals from a liquid phase to a solid phase, generating new hazardous residues. In order to overcome this problem, research should be performed to develop processes for removing heavy metals from effluents followed by its selective recovery. This work is a contribute on the development of an efficient, nearly closed cycle and multi-stage process to remove and recovery selectively heavy metals from electroplating effluents by biosorption and chemical processes.

This thesis is organized in 10 chapters, being the first one a general introduction to the problematic of heavy metals with a review on the literature; this review is basically centred into the use of brewer's yeast strains of *Saccharomyces cerevisiae* on heavy metals removal from industrial effluents and on the main techonologies applied for heavy metals recovery from biomass. Next eight chapters contain a series of papers, elaborated during the PhD, which are published, accepted or submitted for publication.

In Chapter 2, the suitability of using a flocculent brewing yeast strain of *Saccharomyces cerevisiae* in the removal of heavy metals, using flocculation as a separation process, is explored; the influence of cell concentration, presence of different heavy metals and killing temperature on the yeast settling profile was studied. A detailed comparison of the ability of live and dead flocculent cells of *Saccharomyces cerevisiae* to accumulate heavy metals (copper, nickel and zinc) is presented in Chapter 3.

Chapters 4 to 7 are devoted to the optimization and treatment of synthetic and real electroplating effluents by dead biomass. The influence of the matrix of real effluents on the removal of heavy metals by biosorption, as well as the importance of chemical speciation in the prediction and optimization of the treatment of real effluents, where multimetal and multiligands can be present, is explored in Chapter 4. In Chapter 5, the effect of the presence of fluorides on the efficiency of heavy metal removal from electroplating effluents by dead flocculent cells of *Saccharomyces cerevisiae* is studied. In Chapters 6 and 7, the efficiency of dead cells of the brewing strain of *Saccharomyces cerevisiae* NCYC 1364 on the removal of metals from real electroplating effluents, in batch mode, is evaluated. Effluents containing copper, nickel and zinc or chromium, copper and nickel were treated (Chapter 6). Chromium, copper and nickel removal was also assessed without previous reduction of hexavalent chromium to trivalent chromium (Chapter 7).

The feasibility of obtaining a concentrated metals acid solution by incineration of metals loaded biomass and microwave acid digestion of the ashes was assessed in Chapter 8; additionally, metal selective recovery from an acid solution, combining electrochemical and chemical processes, was also studied in Chapter 8 (copper, nickel and zinc) and Chapter 9 (copper, nickel and chromium). Finally, the main conclusions of this work are presented in Chapter 10 together with some suggestions of future work.

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Figure captions

M^{n+}. Biomass (yeast cells): B. For simplicity of representation, the charges of the complexes were omitted. Yeast cells are able to remove heavy metals from industrial effluents (reaction 1). The presence of ligands in the effluents (reactions 2 - 6) can reduce the efficiency of the process by competing with biomass for metal removal.

Table captions

Abbreviations

1/n	Freundlich constant (intensity of adsorption)
%SC	% of settled cells
AAS	Atomic absorption spectroscopy
AAS-FA	Atomic absorption spectroscopy with flame atomization
b	Langmuir constant (biomass affinity to the metal)
CDTA	1,2 –Cyclohexanedinitrilotetraacetic acid
C_{eq}	Metal equilibrium concentration
CSTR	Continuous stirred tank reactor
DEAE	Diethylaminoethyl cellulose
DNA	Desoxyribonucleic acid
EAS	Emission atomic spectroscopy
EDTA	Ethylenediaminetetraacetate
E_h	Redox potential
EU	European Union
FTIR	Fourier transform infrared
IC	Inorganic carbon
ISE	Ion-selective electrode
K	Conditional stability constant (biomass affinity to the metal)
L	Free potential binding sites on biomass
LD_{50}	Dose required to kill 50% of initial population of cells
m	*A*mount of added dry weight biomass
M	Free metal ion
ML	Metal-ion complex
MES	2-(N-morpholino)ethanesulfonic acid
NCYC	National collection of yeast culture
NTA	Nitrilotriacetic acid
PBS	phosphate saline buffer
PI	Propidium Iodide
q	Metal specific uptake
q_{max}	Maximum metal uptake capacity
rpm	Rotations per minute
S.	*Saccharomyces*
SEM	Scanning electron microscopy
TC	Total carbon
TOC	Total organic carbon
US-EPA	United States -Environmental Protection Agency
V	Volume of reaction mixture
YEPD	Yeast extract peptone dextrose

Chapter 1

Introduction

1.1. Overview

Unlike organic pollutants, metals and their salts are not degraded or destroyed; they remain forever in nature. Besides the acute toxicity provoked by several metals (Cu, Hg and Cd), even at low concentration (Wang and Chen 2006), heavy metals can be accumulated through the food chain and create a threat to animals and public health, originating a world-wide environmental problem.

Heavy metals pollution can arise from natural (such as leaching of metals from the rocks) and anthropogenic sources, being the last one the main cause due to industrialization and a matter of concern particularly in developed countries. The main industrial sources of heavy metals are:

i) mining and metallurgical industries (Fe, Zn, Cu, Pb, Cd, Al, Mn, Ni and As) (Kuyucak 2006);
ii) electroplating and other surface finishing industries (Cd, Cr, Cu, Au, Ni, Pt, Ag, Co, Sn) (Schlesinger 2004);
iii) leather and tanning industries (Cr, Al) (Bosnic *et al.* 2000);
iv) energy production (Cu, Cd, Mn, Zn) (Volesky 2001);
v) pigment manufacturing industries (Pb, Cd) (Malakootian *et al.* 2009);
vi) battery manufacturing (Pb, Cu, Cd, Zn, Ni, Cr) (Wasay *et al.* 2001).

Since conventional wastewater treatment plants are not designed to treat toxic effluents, as it is the case of effluents containing heavy metals, a pre-treatment step is necessary for previous removal of heavy metals. Several methods have been used to remove heavy metals, which can be divided in two main groups: chemical

(precipitation/neutralization) and physical methods (ion exchange resins, membrane technologies and activated carbon adsorption). The application of physicochemical methods for the treatment of effluents containing metals has shown problematic since they are not fully efficient (precipitation/neutralization) or present very high costs (membrane technologies and ion exchange resins) when applied to large volumes of wastewater containing low metals concentration (Wang and Chen 2006). The conventional physicochemical methods that are used to treat metal-bearing effluents were detailed described in a recent review (Naja and Volesky 2010).

Alternative processes for detoxification of metal-bearing wastewaters using biological materials have been investigated. The potential of metal concentration by certain types of biomass (algae, bacteria, filamentous fungus and yeast) provides the basis for the development of a new approach to remove heavy metals when they occur at low concentration (Wang and Chen 2009). Among the different types of biomass, yeast cells seem particularly suited for the use in the bioremediation of heavy metals since they may be obtained in large quantities, are an inexpensive source of biomass and have the ability to accumulate a large range of heavy metals, namely Ag^{2+}, Cd^{2+}, Pb^{2+}, Hg^{2+}, Cr^{6+}, Cr^{3+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Sr^{2+} and Zn^{2+} (Al-Saraj *et al.* 1999; Soares *et al.* 2002; Goyal *et al.* 2003; Özer and Özer 2003; Vasudevan *et al.* 2003; Ferraz *et al.* 2004; Han *et al.* 2006; Marques *et al.* 2007; Padmavathy 2008; Cojocaru *et al.* 2009; Chen and Wang 2010), presenting a high potential of application in wastewater treatment.

After effluent treatment, it is of major importance to separate metals from biomass having as objective to recycle biomass and recover metals; this leads to energy savings and consequent reduction of process costs. Desorption studies of heavy metals from yeast biomass using eluants as mineral and organic acids or complexing agents have been performed by several authors (Strandberg *et al.* 1981; Wilhelmi and Duncan 1996; Ferraz *et al.* 2004); however, the solutions obtained

present low metal concentrations, which limits the metal selective recovery process. Concentrated solutions can be obtained after incineration of yeast biomass.

Heavy metals selective and sequential recovery is a big challenge and few studies have been performed. New environmental friendly technologies for selective separation of metals should be developed assuring that some aspects are attended, namely: (i) present high metal recovery, (ii) admit metal recycling, (iii) minimize waste production and (iv) assure low operating costs and easy operation conditions.

The successful use of yeast biomass in bioremediation of metal bearing effluents and consecutive metal selective recovery and recycling depend on the research and development efforts to be carried out in this area.

1.2. Heavy metals removal

1.2.1. *Mechanisms of heavy metals removal by yeast cells*

Metal accumulation by yeasts typically occurs in two steps: firstly by a metabolism-independent process (biosorption) and in the case of live cells, in a second step, by a metabolism-dependent process (bioaccumulation).

1.2.1.1. Biosorption

The first stage on metal accumulation by yeasts, known as biosorption, is fast (occurs in the first minutes of contact with the metal) and takes place in live or dead cells (Blackwell *et al.* 1995). Heavy metals biosorption does not require energy and is not affected by the presence of metabolic inhibitors or by temperature (over a range of 5-30 ºC); this process corresponds essentially to metal binding to yeast surface,

mostly likely to the wall (Blackwell *et al.* 1995). In the case of dead (non-viable) cells, this is, theoretically, the only metal accumulation process due to the lack of metabolic activity. However, metals intracellular accumulation can also occur, by a metabolic independent process, in the case of disruption of yeast cell membrane. The loss of membrane integrity can allow the exposition of further metal-binding sites inside the cells as it is discussed in Chapter 3.

1.2.1.1.1. *Yeast cell wall*

The *Saccharomyces cerevisiae* cell wall, representing 15-25% of the cell dry weight, is a complex macromolecular structure composed mainly by polysaccharides (80-90%) [α-mannans (binded with proteins and phosphate groups) and β-glucans], proteins (5-10%) and chitin (1-2%) (Klis *et al.* 2002). Cell wall provides osmotic and physical protection, acts as a selective permeability barrier, immobilized enzyme support and allows cell-cell recognition and adhesion (Stratford 1994). Cell wall presents a laminated and layered organization constituted by an amorphous inner layer and a fibrilar outer layer. The inner layer is mainly composed by β-glucans and chitin. β-glucans are constituted by fractions of β(1→3) and β(1→6)-linked glucose residues; chitin is composed by linear chains of β(1→4)-linked N-acetylglucosamine (Klis *et al.* 2002). Chitin is present at small amounts in the wall predominantly located in the bud scars (Cabib *et al.* 2001). The outer layer consists predominantly of α-mannans highly glycosylated and associated with proteins (mannoproteins), which corresponds to 30-50 % of wall mass (Cabib *et al.* 2001; Lesage and Bussey 2006); this kind of composition and structure exposes abundant metal-binding functional groups, making cell wall negatively charged.

1.2.1.1.2. *Mechanism of biosorption*

The outer layer of the cell wall, mannoproteic, seems to be more important than the innermost layer, of glucans, in metal accumulation (Brady *et al.* 1994). The blocking of amino, carboxyl or hydroxyl groups of the cell walls reduces the accumulation capacity of Cu^{2+}, which clearly indicated the participation of these functional groups in Cu^{2+} binding (Brady and Duncan 1994a). Wang (2002) and Han *et al.* (2006) reinforced these results showing that esterification of carboxylate functionalities and methylation of amino groups of the cell wall of *S. cerevisiae* resulted in a marked decrease in copper uptake. Also, Lin *et al.* (2005) showed that hydroxyl groups of saccharides and carboxylate anions of aminoacids residues seem to be sites for Au^{3+} binding. Carboxyl, phosphate and amino groups and lipids contribute mostly to lead biosorption (Parvathi *et al.* 2007a). In summary, several groups, namely carboxyl, amine, alcohol, phosphate and sulfhydryl groups, are most likely involved in superficial metal accumulation.

Biosorption of metal ions may take place by different passive metal sequestering processes such as ion exchange, physical adsorption, complexation and microprecipitation; it is possible that some of these mechanisms occur simultaneously, in several degrees, depending on biosorbent and on the solution that contains the metal (Gadd 1990; Volesky 1990). Dai *et al.* (2008) showed that complexation (chemical chelation) is the main mechanism of cadmium biosorption by *S. cerevisiae*; physical adsorption is also involved and is associated with electrostatic attraction, hydrogen bonding and van der Waals forces. Electrostatic attraction (physical adsorption) and complexation also seem to be the most important mechanisms of lead biosorption by yeast (Parvathi *et al.* 2007a). Physical adsorption is also reported as Ni^{2+} and Cr^{6+} mechanism of biosorption by *S. cerevisiae* (Özer and Özer 2003).

Yeast cell wall contain polysaccharides and bivalent ions exchange with counter ions (K^+, Na^+, Mg^{2+}, Ca^{2+}) of these macromolecules. Chen and Wang (2007) hypothesized that this mechanism, ion exchange, was involved on zinc biosorption by *S. cerevisiae*. Other authors have also related the same mechanism for zinc, copper and lead accumulation by *S. cerevisiae* (Brady and Duncan 1994c; Can and Jianlong 2008; Dong *et al.* 2009).

Microprecipitation as a mechanism of biosorption is a consequence of the interaction between metal and cell surface; however, it can be dependent of metabolism as a defensive reaction of cell that produce compounds which facilitate precipitation. Cho and Kim (2003) reported that lead biosorption mechanism by the yeast *Rhodotorula glutinis* involved ion exchange and precipitation by phosphate released from the cell wall or from the cell interior as result of wall damage during drying and griding of biomass.

1.2.1.2. Bioaccumulation

Bioaccumulation is a slow process, dependent on the energy presence and is influenced by temperature, presence of metabolic inhibitors and glucose analogues (Blackwell *et al.* 1995). Bioaccumulation is usually associated to the passage of metal through the cell membrane and subsequent intracellular accumulation (Norris and Kelly 1977; Borst-Pauwels 1981). In yeasts, large amount of metal can be accumulated by this mechanism (Norris and Kelly 1977; Avery and Tobin 1992).

1.2.1.2.1. *Mechanism of bioaccumulation*

In the case of living cells beyond biosorption, bioaccumulation may occur.

As a rule, divalent cations appear to enter in cell, as a result of an electrochemical gradient generated by the activity of H^+-ATPase from plasmatic membrane (Borst-Pauwels 1981; Gadd 1993). Metal accumulation by yeast cells in the presence of glucose can be three times higher than in its absence (Norris and Kelly 1977; Avery and Tobin 1992; Blackwell *et al.* 1995); however, glucose analogues, although absorbed by the cell, do not stimulate metal accumulation; this fact indicates that metal bioaccumulation is not associated to substrate transport across membrane (White and Gadd 1987).

1.2.3. *Factors influencing heavy metals removal*

Metal accumulation is strictly dependent on pH, since it can change metal speciation and availability, as well as the chemistry of biomass and the competition between metal ions. A reduction of cations accumulation with pH decreasing can be attributed to an increase on competition between protons and metals for binding sites (Fourest and Roux 1992). At high pH, metal hydroxides are formed, reducing the free metal available for binding with biomass (Blackwell *et al.* 1995). In the case of hexavalent chromium, metal accumulation increases with pH decreasing (Parvathi and Nagendran 2007) since metal species in solution are anionic. The dominant species in solution is $HCrO_4^-$ at low pH (around 2) and CrO_4^{2-} at higher pH (see Chapter 7). On the other hand, at very low pH values (pH < 3), the biosorbent groups are protonated. As a consequence, the surface of biosorbent will be surrounded by H^+ ions (Parvathi and Nagendran 2007), which enhance the Cr^{6+} interaction with binding sites of the biosorbent due to electrostatic forces (Özer and Özer 2003).

Metal speciation determines metal solubility and availability; this is an important factor for a successful metal biosorption, which is discussed in Section 1.2.4.

The presence of other cations can also interfere in metal accumulation by competition for biomass binding sites (Ting and Teo 1994; Blackwell *et al.* 1995). Ferraz and Teixaira (1999) showed that in lead and chromium mixture solutions, metals compete for yeast binding sites, being this competition pH-dependent. A reduction of chromium accumulation was observed since *S. cerevisiae* seemed to present a higher affinity for lead.

Growth and nutritional conditions, as well as cell age, also influence metal biosorption; distinct conditions imply different cell size (surface area for metal binding), wall composition and extracellular products (Gadd 2009). Goyal *et al.* (2003) has shown that metal biosorption (Cr^{6+} and Fe^{2+}) by *S. cerevisiae*, previously grown in media supplemented with cysteine, glucose, ammonium sulphate, phosphate or ammonium chloride, improve metal uptake; a higher uptake was obtained for cysteine. These nutrients originate different functional groups on yeast cell wall; cysteine introduces protein and sulphydril groups on dead and live cells usually involved on metal binding (Simmons and Singleton 1996).

Additionally, certain treatments can also increase metal accumulation capacity of biomass. Several killing treatments with alkaline solutions, detergents, ethanol, heat drying or the modification of surface biomass with cetyl trimethyl ammonium (Gadd 1990; Avery and Tobin 1992; Lu and Wilkins 1996; Ashkenazy *et al.* 1997; Bingol *et al.* 2004; Göksungur *et al.* 2005) can be used to increase the adsorption capacity of cells of *S. cerevisiae*. Powdering of dried biomass can also expose additional binding sites (Gadd 1990).

The initial concentration of metals can alter the metal accumulation since ion removal is strongly dependent of concentration. Initial concentration acts as a driving force to overcome mass transfer resistance for ion transport between solution and biomass binding site. Biosorption capacity is enhanced by a high initial metal concentration (Wang and Chen 2006).

Temperature usually presents small effects on biosorption. Brady and Duncan (1994b) reported that no pronounced effects are observed on biosorption performance over the range of 5-40 ºC.

1.2.4. *Chemical speciation and heavy metals removal*

Metal chemical speciation is defined as the distribution of a metal among defined chemical species (different chemical forms of a metal or its compounds) in a particular sample or matrix (Templeton *et al.* 2000). This is crucial on heavy metals removal since it determines the metal availability to be sorbed by cells and, as consequence, the biosorption efficiency.

Chemical speciation is influenced by pH, redox potential (E_h), metals concentration and organic/inorganic ligands concentration, as well as their affinity for metal ions (Hughes and Poole 1991). At acidic pH values, metals exist as free cations and are available for biosorption. However, hydrogen ion concentration is also increased and hence possible competition with metal ions for the available binding sites occurs. Increasing pH leads to precipitation as insoluble hydroxides, lowering its availability for biosorption (Gadd 1993; Blackwell *et al.* 1995). Redox potential can also affect metal speciation; for example, chromium exists as Cr^{6+} or Cr^{3+} according to the E_h of the solution. The presence of inorganic anions (e.g. carbonates, chlorides, fluorides, phosphates and sulphates) in solution can also affect species distribution through complexation with metals reducing metal availability to be adsorbed by biomass (Hughes and Poole 1991; Avery and Tobin 1993; Gadd 1993). For example, lead associates with inorganic ligands such as ferric hydroxide and silica reducing its availability for biomass adsorption (Florence 1982). Organic compounds can also decrease heavy metals removal. Florence (1982) reported that copper tends to form complexes with organic ligands having sulfhydril or sulfur groups not being available for adsorption by biomass. Besides the metals of interest,

the presence of other cations in solution can reduce heavy metals accumulation by competition for the binding sites of biomass, as it was reported above (Section 1.2.3).

Metal chemical speciation is an important tool on the optimization of heavy metals removal. Considering a defined matrix (e.g. an electroplating effluent) and its constitution (ligands, metals, pH), it is possible to predict the availability of metals in solution for being removed by the biomass. Additionally, it is possible to adjust matrix parameters to increase metal availability and consequently increase metal removal from solution, as it will be shown in Chapter 4.

1.2.5. *Evaluation of heavy metals removal performance*

Adsorption isotherms have been used to assess metal accumulation capacity by several types of microorganisms, including yeasts. However, it should be taken in mind that isotherm models of Langmuir and Freundlich were originally developed to more defined systems, such as gas adsorption to activated carbon. Other adsorption isotherms, like Brunauer-Emmett-Teller model (BET), are just mathematical models capable of describing the relation between metal uptake and final equilibrium concentration of the residual metal in solution. So, these models are quite simplistic when applied to complex biological systems, as yeast cells. As stated above, it is difficult to determine the importance of each ligand in the process of bioremediation; in addition, it is unlikely that these systems present a true monolayer of adsorption or that no movement of molecules on the biological surface material occurs. Despite the limitations indicated, isotherms provide important information about heavy metals accumulation capacity by different types of biomass.

Different models of adsorption isotherms have been proposed in the literature, being Langmuir and Freundlich models commonly used for monolayer adsorption.

BET isotherms reflect multilayer adsorption, reducing, in the limit, to Langmuir model, when adsorption occurs in monolayer (Volesky 2003b).

The general equation of Langmuir model equation is:

$$q = q_{max}\, b\, C_e / (1 + b\, C_{eq}) \qquad [1\text{-}1]$$

where q is the metal uptake capacity (mg or mol of metal/g dry weight biomass), q_{max} is the maximum metal uptake capacity (mg or mol of metal/g dry weight biomass), C_{eq} is the final concentration (concentration of equilibrium) (mg or mol metal/l) and b is a constant related to adsorption energy, indicating the biosorbent affinity to the metal.

The linearization of the equation [1-1] can be obtained calculating its inverse:

$$1/q = 1/q_{max} + [1/(q_{max}\, b)]\, (1/C_{eq}) \qquad [1\text{-}2]$$

The graphic representation of the inverse of metal uptake capacity $(1/q)$ versus the inverse of the final concentration $(1/C_{eq})$ enables to determine the values of q_{max} and of b from the intercept and the slope of the equation [1-2].

The general equation [1-3] of Freundlich model is:

$$q = k C^{1/n} \qquad [1\text{-}3]$$

this equation can be easily linearized, equation [1-4], by applying logarithms to both sides of the equation:

$$\ln q = \ln k + (1/n) \ln C_{eq} \qquad [1\text{-}4]$$

Similarly to the treatment described for the Langmuir model, representing *ln q* due to *ln* C_{eq} allows to determine the uptake capacity *(k)* and the intensity *(1/n)* of adsorption from the intercept and the slope of the equation; q and C_{eq} have the same meaning as in the Langmuir model.

Maximum biosorption capacity and the affinity of the biosorbent material for a given metal are two important aspects that should be taken into account in the characterization of a given biomass. An ideal biosorbent should display a high value of q_{max} (high load capacity) and low values of b (high biomass affinity for metal) in the Langmuir equation.

BET isotherm assumes that a Langmuir isotherm is applied to each layer and can be represented by the following equation [1-5]:

$$q = bC_{eq}\, q_{max} / \, [(C_{sat} - C_{eq})\, [1 + (b-1)\, C_{eq} / \, C_{sat}]] \qquad [1\text{-}5]$$

where C_{sat} is the saturation concentration (mg or mol metal / l); q, b, C_{eq} and q_{max} have the same meaning as in the Langmuir model.

The linearization of the equation [1-5] can be obtained calculating its inverse:

$$C_{eq} / [(C_{sat} - C_{eq})\, q] = 1 / (b\, q_{max}) + [(b-1) / \, b\, q_{max}]\, (C_{eq} / \, C_{sat}) \qquad [1\text{-}6]$$

The graphic of $C_{eq} / \, [(C_{sat} - C_{eq})\, q]$ against $C_{eq} / \, C_{sat}$ gives a straight line that allows to determine the values of q_{max} and of b from the intercept and the slope of the equation [1-6].

Ruzic and Scatchard linearization methods have also been used to determine conditional stability constant for the formation of metal-ligand. In these models, it is

assumed that the complexes formed between the metal and the binding sites on biomass have 1:1 stoichiometry, according to the following equation, [1-7]:

$$M + L \leftrightarrows ML \qquad [1\text{-}7]$$

where *M* is the metal ion in consideration, *L* is the free ligand and *ML* is the metal-ion complex. A conditional stability constant, *K* is defined by:

$$K = [ML]/[M][L] \qquad [1\text{-}8]$$

being *[ML]*, *[M]* and *[L]* the molar concentration of metal-ion complex, free metal ion and free ligand (biomass).

Combining the equilibrium constant expression with the material balances of the binding sites of biomass and metal ion, Ruzic and Scatchard equations, [1-9] and [1-10] respectively, are obtained. Ruzic equation can be represented as follows:

$$[M]/[ML] = 1/(K\,[L]) + (1/[L])[M] \qquad [1\text{-}9]$$

The plot of *[M]/[ML]* against *[M]* displays a straight line, which allows to determine *[L]* and *K* from the slope and the intercept of the equation.

Scatchard equation can be represented as follows:

$$[ML]/[M] = K\,[L] - K\,[ML] \qquad [1\text{-}10]$$

The plot of *[ML]/[M]* against *[ML]* displays a straight line, which allows to determine *K* and *[L]* from the slope and the intercept of the equation.

Total concentration of ligand and conditional stability constant, *K,* (biomass affinity for metal) are fundamental for the optimization of the biosorption process.

Kinetics studies are also important in order to assess the temporal evolution of metal accumulation by biomass; these studies allow establishing the equilibrium time on adsorption isotherms. A rapid metal accumulation by the biosorbent is desirable in order to provide a short contact time between metal bearing solution and biosorbent material.

In conclusion, kinetic and equilibrium studies are crucial for a quantitative assessment of heavy metals accumulation characteristics of a specific type of biomass. In Chapter 3 (Section 3.4, Table 3.2) are presented metal accumulation capacities and operating conditions (pH and contact time) of equilibrium and kinetic studies for different types of *S. cerevisiae* described on the literature.

1.2.6. *Live versus dead cells in heavy metals removal*

The use of dead cells on metal removal has some advantages over live cells. Dead biomass is immune to the toxic effects of heavy metals, independent on nutrients supply, less affected by extreme operating conditions of pH and temperature and ease to store (Kapoor and Viraraghavan 1995). Further, metal and biomass recovery is easier for dead biomass, since intracellular metal accumulated by live biomass can only be recuperated by destructive processes (Bishnoi and Garima 2005). Live cells can drastically decrease during heavy metals elution; as a consequence, alterations on process efficiency can occur (Suh *et al.* 1998). However, in some cases, live yeast biomass can accumulate higher amounts of metal than dead one due to intracellular accumulation, being desirable its application on heavy metals removal from effluents (Ross 1977; Stoll and Duncan 1996; Blackwell and Tobin 1999).

Several methods have been described for inactivating microbial biomass such as sterilization, heat treatment or chemical treatment (with acids or basis). The way how cells are inactivated affects its metal accumulation capacity, being heat treatment the technique with better results (Tsezos 1990). So, killing/inactivating treatment of biomass determines metal-binding capacity and can be adjusted to improve metal binding capacities and affinities.

The biomass cost is another important factor. Dead yeast biomass can be obtained inexpensively as a by-product of industrial processes, that presents disposal problems (as described above) (Wang and Chen 2006). Thus, dead biomass costs can only be associated to its transport and drying, if necessary.

1.2.7. *The problem of biomass separation*

The industrial use of microbial biomass on metal removal has some limitations, which are consequence of their small size, strength and density; these characteristics, inherent to the nature of biomass, theoretically, limit the type of reactor to use and difficult the separation process of treated effluent (Tsezos 1990).

1.2.7.1. Biomass immobilization

The difficulty of applying biomass on effluents treatment has been overcome by using several immobilization techniques. Thus, several studies have been performed on removal of metals by immobilized strains of *S. cerevisiae*. Wilheimi and Duncan (1995) studied copper, zinc, cobalt, nickel and chromium chlorides accumulation by immobilized *S. cerevisiae* in packed-bed continuous flow columns. Zhao and Duncan (1997) reported an high efficiency on copper, zinc and cadmium removal from aqueous solutions by a strain of *S. cerevisiae* cross-linked with

formaldehyde in a column bioreactor. A brewer's yeast of *S. cerevisiae* immobilized on a polyvinyl alcohol matrix was also applied on copper uptake (Ting and Sun 2000). More recently, a strain of *S. cerevisiae* immobilized on the surface of a new adsorbent of chitosan-coated magnetic nanoparticles was applied on copper removal from aqueous solutions (Peng *et al.* 2010).

Immobilized biomass can be used in fixed bed or fluidized reactors of high density. Nevertheless, cell imobilization presents some contraints: has mass transfer limitations, reduces cell viability, is unsuitable at high pH, can decrease metal accumulation and can even present prohibitively costs of biossorbent preparation (Cassidy *et al.* 1996; Wang and Chen 2009).

1.2.7.2. Flocculation as an alternative separation process

Some brewing yeasts (ale and lager strains) have the ability to form clumps in absence of sugars. Traditionally, ale strains form flocs that rise to the top of the liquid and flocs formed by lager strains fall to the bottom of the liquid; both strains leave aqueous solution clear (Verstrepen *et al.* 2003). This natural, asexual and reversible aggregation, known as flocculation, enables an efficient separation of cells from the aqueous solution where cells are suspended.

Flocculation of yeast cells is a complex process that depends of several factors: yeast strain (genetics, physiological state and metabolism), culture media composition and growth conditions (temperature, pH, agitation and oxygen content) (Verstrepen *et al.* 2003). According to the most generally accepted mechanism of flocculation, yeast aggregation involves the presence of specific proteins (lectins), which recognize and interact with carbohydrate residues (mannan receptors) on neighbouring cell walls (Miki *et al.* 1982). The presence of calcium ions is required for lectins activation.

Thus, flocculent yeast cells might be used as a useful, cheap, efficient and environmental-friendly process of cell separation and recovery from aqueous solutions. Several studies using flocculent cells of *S.cerevisiae* on heavy metals removal have been described (Avery and Tobin 1992; Ferraz and Teixeira 1999; Marques *et al.* 1999; Marques *et al.* 2000; Ferraz *et al.* 2004). Soares et al.(2002) have shown that a flocculent strain of *S. cerevisiae* presents a higher copper uptake than its isogenic non-flocculent strain; since heavy metals occupy lectin-calcium binding sites, this fact improves metal binding sites and consequently metal accumulation. So, brewer's yeast cells seem to present a high potential of application on metal removal since its natural aggregation particularity allows combining metal removal with an efficient biomass separation.

Additionally, flocculent yeast application on effluents treatment enables the use of different reactors configuration without the risk of biomass washout; these reactors can be employed on a large scale with low operation and maintenance efforts and do not require an energy input for cell separation.

1.2.8. *Examples of bioreactors configurations*

Batch or continuous operating systems can be applied on metals removal by biosorption. The choice of batch or continuous operating mode is dependent of several factors like physical characteristics of biossorbent, hydraulic flow, space availability and investiment costs (Park *et al.* 2010).

Different reactor configurations have been proposed for metals removal, namely stirred tank reactors, up-flow and down flow packed bed reactors, fluidized bed reactors, pulsating bed reactors, multiple-bed contactors and air-lift reactors (Tsezos 1990; Volesky 1990; Tobin *et al.* 1994; Gavrilescu 2004; Das *et al.* 2008).

In the case of agitated reactors, operating in batch mode, after an appropriate time of contact of metal (determined by kinetics of accumulation) with biomass occurs a step of solid-liquid separation (by sedimentation, flotation, centrifugation, filtration or flocculation), resulting in an effluent containing a low amount of heavy metals and biomass loaded with metals. Metal bearing biomass can subsequently be regenerated, incinerated (or dissolved) or packaged conveniently (Volesky 1990; Tobin *et al.* 1994).

For continuous reactors, effluent treatment can operate by two alternative processes: a continuos supply of the effluent to be treated and of the fresh/regenerated biomass or using a biosorbent batch; in the first case, the biomass separation from effluent is continuous, while in the second case the feeding is continuous with metal bearing effluent until biomass bed is saturated (Volesky 2003a).

Stirred batch tank reactors are rarely used on industrial applications due to the problems associated with solid-liquid separation. However, if the retention time necessary to remove metals is high, stirred batch tank is the most economic alternative (Park *et al.* 2010).

Between continuous reactors, the up-flow packed bed reactor, commonly known as column reactor, has been the most applied in biosorption studies due to its high efficiency and easy scale up (Park *et al.* 2010). Its application is conditioned by the regeneration of the column for the next sorption cycles (Volesky 2001).

1.2.9. *Examples of biomass application in the treatment of real effluents*

Biosorption using yeast biomass, namely cells of *S. cerevisiae*, can be a good replacement of conventional technologies for the treatment of metal-loaded effluents

or can be applied as a polishing step after an initial treatment (for example, after alkaline precipitation not completely efficient). However, despiting big research efforts to study the influence of several factors on metal biosorption by yeast biomass in synthetic solutions, few studies have been performed in order to treat real effluents.

Stoll and Duncan (1996) studied the removal of metals (Cu^{2+}, Cr^{6+}, Cd^{2+}, Ni^{2+} and Zn^{2+}) from electroplating effluents by viable *S. cerevisiae* and the effect of glucose treatment on accumulation efficiency. Immobilized non-viable cells of *S. cerevisiae* were also applied to remove Cu, Cd, Cr, Ni and Zn from electroplating effluents in continuous-flow stirred reactors. After treatment, metals concentration was above the stipulated drinking water quality criteria (Stoll and Duncan 1997).

Biosorption of Cr^{6+} ions from a real electroplating effluent and from aqueous synthetic solution with dead fungal biomass (*Aspergillus niger*, *Aspergillus sydoni* and *Penicillium janthunellum*) was investigated in batch mode and compared. Cr^{6+} removal from electroplating wastewater was less than from synthetic solution (Kumar *et al.* 2008). A mutant strain of *S. cerevisiae* was also used to evaluate the ability to remove Mn^{2+}, Cu^{2+}, Co^{2+}, and Cd^{2+} from synthetic effluents, being more efficient than the wild-type to remove the first three metals (Ruta *et al.* 2010).

Other studies using real effluents to optimize biosorption conditions have been reported. Parvathi *et al.* (2007a) investigated the potential of waste beer yeast *S. cerevisiae* for biosorbing lead from battery manufacturing industrial effluent. Parameters, like biomass concentration, pH of effluent and agitation, were optimized and lead uptake capacity determined. The effect of pH on chromium biosorption by chemical treated *S. cerevisiae* in tannery effluents was also studied (Parvathi *et al.* 2007b). Biosorption optimization of chromium from chrome-electroplating industry using waste yeast biomass of *S. cerevisiae* was also carried out (Parvathi and Nagendran 2007).

The application of microorganisms on the removal of heavy metals from real effluents is a hard task since it involves the integration of different fields of knowledge, such as microbiology, biochemistry, analytical chemistry and chemical engineering. It is clear that yeast biomass can be competitive with existing treatments but further studies are needed in order to exploite all potentialities of this biomass on heavy metals removal from industrial effluents.

1.2.10. *Biomass regeneration*

One of the foccus regarding the use of microbial biomass in biotechnology applications on the removal of heavy metals from industrial effluents is the possibility of its reuse and heavy metals recovery. The process of recovery depends on how easy it is to remove the metals from biomass, which in turn depends first of the metal species involved and secondly of the mechanism of accumulation.

In the case of heavy metals accumulation by biosorption, biomass can be theoretically regenerated and heavy metals recovered by a desorption process. Conversely, in the case of bioaccumulation of heavy metals, biomass can not be regenerated since metals recovery requires a destructive procedure (dissolution with strong acids or basis or by incineration) due to intracellular accumulation of the metals.

Research studies have been concentrated in non-destructive processes of biomass desorption and subsequent regeneration. The elution process should be efficient (maximum recovery with the smallest volume of eluent), not cause damage to biomass (to assure that accumulation capacity does not decrease during the sucessives biosorption/desorption cycles) and be economic. Several acids (HCl, HNO_3 and H_2SO_4), basis (NaOH, Na_2CO_3) or diluted (0.1 M) complexing agents

(EDTA) have been shown effective on metal elution (Bai and Abraham 2003; Ferraz *et al.* 2004; Padmavathy 2008; Cui *et al.* 2010).

Nevertheless, non-destructive processes of heavy metals recovery from biomass present some limitations namely the decrease of biomass accumulation capacity during the sucessives cycles and a solution with low metals concentration is obtained.

1.3. Heavy metals selective recovery

Heavy metals selective recovery from biomass allows metals recycling and contributes to reduce the costs since the metals can be reintroduced in the industrial process or re-sailed. However, industrial effluents commonly contain multiple heavy metals which difficults its selective removal.

Some studies have been performed for recovering selectively heavy metals (more than two) (Tabak *et al.* 2003; Fukuta *et al.* 2004; Tokuda *et al.* 2008; Kuchar *et al.* 2010; Soya *et al.* 2010). The selective recovery of copper, nickel and zinc by sequential precipitation using sulphides was investigated by Fukuta *et al.* (2004) and Tokuda *et al.* (2008) with relatively high selectivity efficiency. Recently, Soya *et al.*(2010) treated a simulated plating solution containing copper, nickel and zinc with CaS. Selective precipitation of metals sulfides was obtained at different pH values with a selectivity of 99.5 and 81.4% for copper and zinc, respectively, remaining nickel sulfide in the slurry.

Kuchar *et al.* (2010) have also recovered selectively copper, nickel, zinc and chromium by combining sulfidation and oxidation treatments. In sulfidation treatment, selective precipitation of 96.6 and 91.5% of Cu (pH 1.5) and Zn (pH 4.5)

were achieved, respectively. In the oxidation treatment, only a selectivity of 64% was obtained for nickel since the oxidation of chromium was inneficient.

However the use of sulphides implies the application of adequate safety procedures, since it is toxic and corrosive. In addition, the transport, storage and production of sulphides are expensive (Huisman *et al.* 2006). New technologies should be developed in order to create economic and environmentally friendly processes for selective and effective metal recovery.

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Chapter 2

Removal of heavy metals using a brewer's yeast strain of *Saccharomyces cerevisiae*: the flocculation as a separation process[1]

Abstract

In this work, a brewer's yeast strain was used to remove heavy metals from a synthetic effluent. The solid-liquid separation process was carried out using the flocculation ability of the strain. The yeast strain was able to sediment in the presence of Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+} and Cr^{3+}, which evidences that flocculation can be used as a cheap and natural separation process for an enlarged range of industrial effluents. For a biomass concentration higher than 0.5 g/l, more than 95% of the cells were settled after 5 minutes; this fact shows that the auto-aggregation of yeast biomass is a rapid and efficient separation process. Cells inactivated at 45 ºC retain sedimentation characteristics, while inactivated at 80 ºC lose partially (40%) the flocculation. The passage of metal-loaded effluent through a series of sequential batches allowed, after the second batch, the reduction of Ni^{2+} in solution for values below the limit of discharge of wastewater in natural waters (2 mg/l); this procedure corresponds to a removal of 91%. A subsequent batch had a marginal effect on Ni^{2+} removal (96%). Together, the results obtained suggest that the use of brewing flocculent biomass looks a promising alternative in the bioremediation of metal-loaded industrial effluents since the removal of the heavy metals and cell separation are simultaneously achieved.

[1] Bioresource Technology 99 (2008) 2107-2115

2.1. Introduction

Large amount of heavy metals are released into the environment due to the technological activities of humans. The impact of these metals in aquatic systems and their accretion throughout the food chain can cause a serious threat to animals and humans, originating a world-wide environmental problem. The currently technologies (such as, precipitation, ion exchange and reverse osmosis) for removal of heavy metals from industrial effluents appear to be inadequate or expensive; alkaline precipitation often creates secondary problems with metal-bearing sludges (Volesky 1990; Volesky 2001).

The potential of metal concentration by certain types of biomass provides the basis for the development of a new approach to remove heavy metals when they occur at low concentration (Volesky 1990; Vieira and Volesky 2000; Volesky 2001). Some types of industrial fermentation waste biomass are excellent metal sorbers. Yeast cells may be obtained in large quantities and are an inexpensive source of biomass (they are wastes from wine and brewing industry), with an ability to accumulate a broad range of heavy metals under a wide range of external conditions (Blackwell *et al.* 1995). Thus, they constitute a wastewater treatment alternative where cost effectiveness is the main attraction.

A difficulty of applying microbial biomass for heavy metals remediation lies with the rapid and efficient separation of the biomass from the reaction mixture after contact (Tsezos 1990). Different solid-liquid separation techniques can be used such as centrifugation or filtration. Centrifugation is considered as a relatively expensive method, regarding the power demand per quantity of microorganisms recovered, while filtration may also face blocking problems of the filters, especially with ultra-fine size particles, like bacteria or yeast cells, making these processes unfeasible in remediation technologies. Another possibility can be the use of different immobilization techniques namely, gel entrapment (such as in calcium alginate),

adsorption to an inert support (like DEAE cellulose) or entrapment in a preformed carrier (such as porous glass) (Norton and D'Amore 1994; Ting and Sun 2000; Brányik *et al.* 2001; Godjevargova *et al.* 2004). Besides the cost of immobilization, these techniques have some disadvantages: mass transfer limitations, unsuitability at high pH, diminution of cell viability due to the immobilization process, decrease in metal adsorption properties of the biomass and poorer kinetic characteristics as compared with the same biomass in a no immobilized form (Tsezos 1990; Cassidy *et al.* 1996). In the last years, flotation has been proposed as a separation technique for different types of particles including several kinds of biomass (Zouboulis and Matis 1997; Lazaridis *et al.* 2004). This cell separation process is relatively fast and efficient. However this process needs the addition of ionic surfactants and the generation of bubbles (which requires energy input) to rise the particules to the surface.

Another possibility may involve the use of flocculating yeasts. Several brewing yeasts have the ability of interact between them and flocculate (Stratford 1992). Thus, the use of flocculent yeast strains can be a promissory alternative, since it is a natural, eco-friendly and cheap process of cell separation and allows recycling the biomass (Figure 2.1).

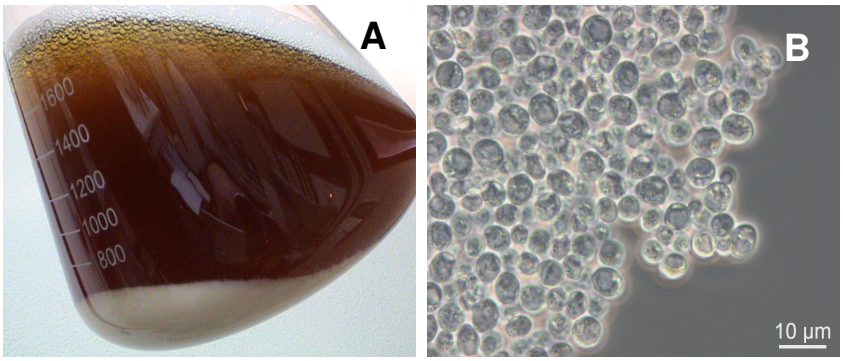

Figure 2.1. Culture of the brewing flocculent strain of *Saccharomyces cerevisiae* NCYC 1364 (**A**). Photomicrograph of phase-contrast observation of the culture (**B**).

According to the lectin-like mechanism, flocculation occurs as a result of the interaction of cell wall proteins (lectins), present only on flocculent cells, with carbohydrates (receptors) on the neighbouring cell wall; calcium is necessary for the activation of the lectins (Miki *et al.* 1982a; Stratford 1992). Yeast flocculation is under genetic control. Dominant and recessive genes and flocculation suppressor genes have been described (Verstrepen *et al.* 2003).

Recently, it was shown that a flocculent strain has a higher Cu^{2+} uptake than the isogenic (except for the marker genes and the gene *FLO1*) non-flocculent strain (Soares *et al.* 2002). It is likely that heavy metals can also occupy lectin Ca^{2+} binding sites with the consequent increase in heavy metals biosorption, since the cell walls of flocculent cells may provide additional metal-binding sites than the non-flocculent ones (Soares *et al.* 2002).

Given the above arguments, the use of flocculating yeasts can be an advantageous alternative, since it is an inexpensive process of cell contained and allows the use of different configurations of suspended biomass reactor without the risk of washout; additionally, flocculent yeasts may have a higher ability of metal accumulation. Even though, brewer biomass has been used in several studies for heavy metals removal (Volesky *et al.* 1993; Volesky and May-Phillips 1995; Simon and Singleton 1996; Blackwell and Tobin 1999; Lin *et al.* 2006), including flocculent ones (Avery and Tobin 1992; Ferraz and Teixeira 1999; Marques *et al.* 1999; 2000; Ferraz *et al.* 2004), the complete exploiting of the characteristics of this kind of biomass was not carried out.

In this work the effect of cell concentration, the presence of different heavy metals, and the killing temperature on the yeast settling profile was studied. Additionally, the suitability of using flocculent cells in the removal of heavy metal from a simulated electroplating effluent has been explored.

2.2. Materials and methods

2.2.1. *Strains, media and culture conditions*

Four flocculent strains of *Saccharomyces cerevisiae* were used in this work. They were the ale-brewing strains National Collection of Yeast Culture (NCYC) 1190, NCYC 1195; NCYC 1364 and the laboratory strain S646-1B (MAT a/α, *HO*/*HO*, *FLO1*/*FLO1*, *ade1*/*ade1*). The original strains were obtained from the NCYC, UK, except the strain S646-1B which was gift from Dr Malcom Stratford. The strains were routinely maintained at 4 ºC on YEPD agar slopes containing (per litre of water): yeast extract, 10 g; peptone, 20 g; glucose, 20 g; agar, 20 g.

Pre-cultures were prepared in 40 ml of YEPD broth in 100 ml Erlenmeyer flasks. Cells were incubated at 25 ºC on an orbital shaker Sanyo Gallenkamp IOC 400 (West Sussex, UK), at 150 rpm, during 24 hours.

Cultures in YEPD with 50 g/l glucose were prepared by inoculating 1 l of culture medium, in 2 l Erlenmeyer flasks, with 4% (v/v) from pre-cultures. Cells were incubated in the same conditions of the pre-culture for 48 hours.

2.2.2. *Preparation of biomass*

After growth, cells were harvested by centrifugation (2000 *g*, 5 minutes), washed two times with 30 mM ethylenediaminetetraacetic acid (EDTA) solution. Subsequently, cells were washed once with deionised water, once with 10 mM [2-(N-morpholino)ethanesulfonic acid] MES pH buffer, at pH 6.0 and resuspended in MES pH buffer. MES is a suitable pH buffer for heavy metal uptake studies because it does not complex with several metal ions, such as cadmium, copper, lead and zinc (Soares *et al.* 1999a; 1999b). Additionally, yeast cells maintain viability and no

detectable amounts of protein or inorganic phosphate are released when incubated in this buffer during 48 h (Soares *et al.* 2000).

Inactivated cells were obtained by drying live biomass at 45, 55 or 80 ºC, until constant weight.

2.2.3. *Measurement of sedimentation ability*

The sedimentation ability was evaluated under standard conditions, using a technique previously described (Soares and Mota 1997). Briefly, cells suspensions were placed in a 25 ml cylinder, at 5 g dry weight/l (unless stated otherwise), in MES pH buffer (10 mM, pH 6.0), containing 4 mM Ca^{2+} (control ion) or industrial effluent adjusted to pH 6.0 with or without the addition of 4 mM Ca^{2+}. Additionally, the sedimentability of the yeast strain was tested in MES pH buffer (10 mM, pH 6.0) in the presence of 0.2 mM of Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+} or Cr^{3+} from metal salt stock solutions of $Cu(NO_3)_2$, $NiCl_2$, $ZnCl_2$, $CdCl_2$ and $Cr(NO_3)_3$. In all cases the suspension was agitated (18 inversions of the cylinder) to promote flocculation. At defined periods of time, indicated in the figures, samples were taken from a fixed position of the cylinder (the level corresponding to 20 ml) and dispersed in 30 mM EDTA solution. Cell concentration after a *t* time (C_t) was determined by measuring the absorbance of the suspension at 600 nm. Calibration curves (absorbance versus either number of cells or dry weight) were previously constructed.

In order to determine the initial cell concentration, cell suspensions were placed in 30 mM EDTA solution, in a 25 ml cylinder. The suspensions were agitated (18 inversions of the cylinder). Samples were taken and diluted in 30 mM EDTA solution, before absorbance was determined at 600 nm (C_i).

The % of settled cells (*%SC*) was calculated by the following equation:

$$\%SC = 100 - (C_t/C_i) \times 100 \qquad [2\text{-}1]$$

where C_i is the initial cell concentration and C_t is the cell concentration in suspension after t time.

In the case of inactivated cells, after drying, the biomass was grounded by using a mortar and pestle. From each sample, a defined amount of biomass was weighted and placed in a beaker with deionised water. The suspension was mixed very well to ensure a complete dispersion of the cells and then the sedimentation ability evaluated as described above.

2.2.4. *Assessing of structural changing of yeast cells*

Cells were concentrated by centrifugation, at 4500 *g*, during 1 minute, washed and resuspended in phosphate saline buffer (PBS) (50 mM, pH 7.4), at $5x10^7$cells/ml. Propidium Iodide (PI) (Sigma, Steinheim, Germany) was added to a final concentration of 3 μg/ml. Cell suspension was incubated at room temperature, in the dark, for 20 minutes. Cells were examined using a Leica DLMB epifluorescence microscope (Leica Microsytems, Wetzlar GmbH, Germany) equipped with a HBO-100 mercury lamp and a filter set I3 (excitation filter BP 450-490, dichromatic mirror 510 and suppression filter LP 515) from Leica. Images were acquired with a Leica DC 300F camera (Leica Microsystems, Heerbrugg, Switzerland) using a N plan x 100 objective; the images were processed using Leica IM 50-Image manager software.

2.2.5. *Measurement of cell volume*

Photos of live and inactivated biomass at 45, 55 or 80 ºC were recorded using a Leica DC 300F camera. In order to achieve accuracy in the measurement of mean cell volume, at least 20 photos were taken in randomly selected fields, using an N plan x 100 objective. A minimum sample size of 300 cells was used for all

measurements. The images were stored and processed with Leica IM 50-Image manager software.

The volume of each yeast cell was calculated based on the assumption that yeast generally conforms to the shape of a prolate ellipsoid. Cell volume is defined as:

$$V = LB^2\pi/6 \qquad [2\text{-}2]$$

where L and B are cell length and width, respectively.

2.2.6. *Bioremediation of wastewater using a batch reaction system*

Inactivated biomass, after being dried at 45 ºC until constant weight, was washed five times with an excess of deionised water.

Cell suspensions with a concentration near of 12 g dry weight/l were added to a synthetic effluent containing Ni^{2+}, Cu^{2+} and Cr^{3+} and shaken in 500 ml plastic flasks at 150 rpm, in an orbital shaker, at 25 ºC. After 30 minutes of contact of the biomass with the effluent, cells were removed and the supernatant added to new yeast biomass, in a subsequent batch. Control experiments were done incubating the synthetic effluents in the absence of biomass, in the same conditions described above; these control experiments showed that no appreciable amount of nickel (less than 3%) was adsorbed to the plastic flasks.

Heavy metals were determined by Atomic Absorption Spectroscopy with flame atomization (AAS-FA) in a Perkin Elmer AAnalyst 400 spectrometer (Norwalk, CT, USA), after appropriate dilution of the samples.

2.3. Results and discussion

2.3.1. *Selection of the flocculent strain*

For comparative purposes four flocculent strains of *Saccharomyces cerevisiae* were used. All of them were able to settle in a wide range of pH (3-9) (Stratford 1989; Soares and Seynaeve 2000). This pH ranges embraces almost all pH values found in industrial effluents. All strains were suspended in the following solutions: (i) effluent adjusted to pH 6.0, (ii) effluent plus 4 mM Ca^{2+} and in (iii) MES pH buffer (pH 6.0, 10 mM) with 4 mM Ca^{2+}; the last solution represents the standard conditions for flocculation evaluation (Ca^{2+} is the control ion). Figure 2.2 shows that all were strongly flocculent (more than 95% of the cells were settled after 20 minutes), in buffer in the presence of Ca^{2+}.

Additionally, all strains were able to settle in the presence of the effluent containing high amount of metals: 2.3 mM Cu^{2+}, 1.5 mM Ni^{2+} and 0.8 mM Zn^{2+}. However, only the strains NCYC 1190 and NCYC 1364 were fully flocculent in the effluent without calcium (Figure 2.2-B and D). For these two strains, the sedimentation, in the effluent without Ca^{2+}, is a fast process, being more than 95% of cells settled after 5 minutes (Figure 2B and 2D). Since the strain NCYC 1364 requires less amount of Ca^{2+} (0.05 mM) to induce a full sedimentation, than the strain NCYC 1190 (0.1 mM) (Soares and Seynaeve 2000), it is likely that this strain also requires less amount of bivalent cations; thus, the strain NCYC 1364 was selected.

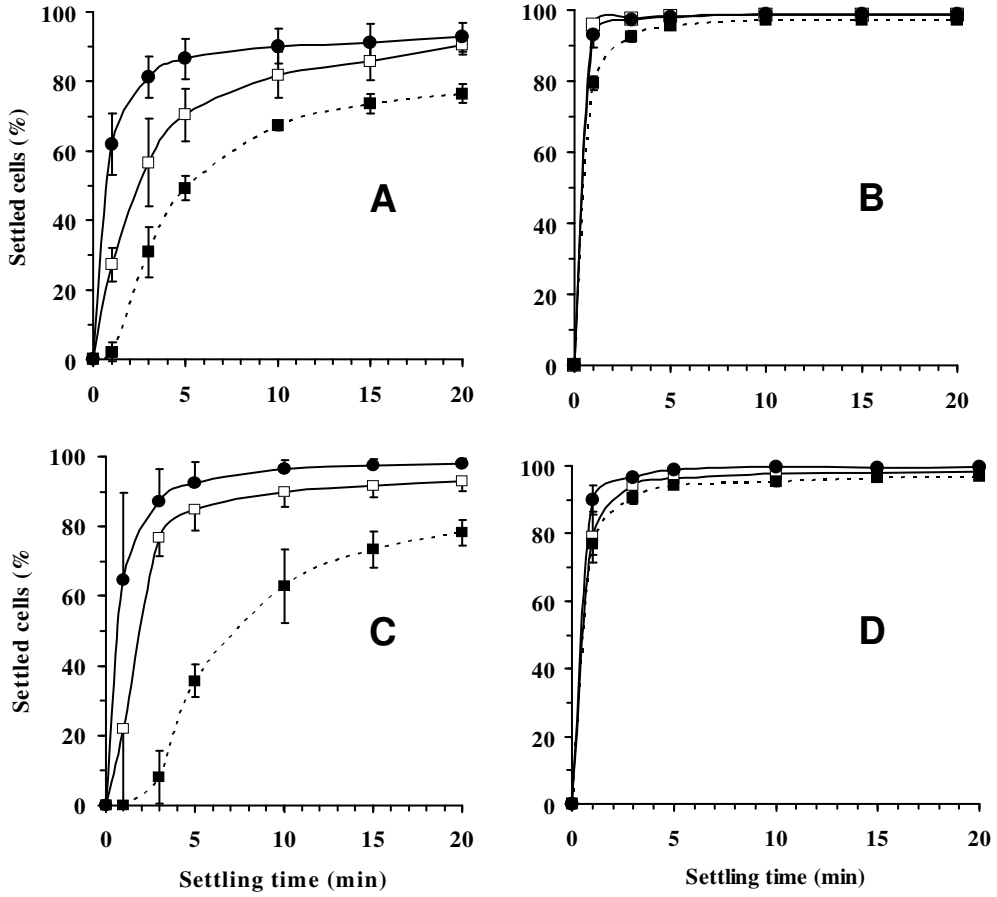

Figure 2.2. Settling profiles of *Saccharomyces cerevisiae* S646-1B (A), NCYC 1190 (B), NCYC 1195 (C) and NCYC 1364 (D). Yeast biomass (5 g dry weight/l) was suspended in: (●) 10 mM MES buffer, at pH 6.0, with 4 mM Ca^{2+} (control); (■) industrial effluent containing 2.3 mM Cu^{2+}, 1.5 mM Ni^{2+} and 0.8 mM Zn^{2+}, adjusted to pH 6.0 or (□) effluent adjusted to pH 6.0 plus 4 mM Ca^{2+}. Values are the mean of three replicates of two independent experiments; standard deviations are presented (n =6).

2.3.2. *Sedimentation in the presence of heavy metals*

To test the flocculation ability, as a separation process in a wide range of effluents containing heavy metals, different synthetic effluents were prepared containing different pairs of the following heavy metals: Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+} and Cr^{3+}. For all, a metal concentration of 0.2 mM was used. Figure 2.3 shows that the strain NCYC 1364 settled fully in any heavy metals combination (the remaining

combinations of metal pairs are presented in Annex A-1 for pictorial clarity). This strain also shows a fully sedimentation ability when resuspended in the synthetic effluent described in the Section 2.3.5, as well as in real electroplating effluents, namely the effluent reported in Chapter 4, Section 4.3.1 and the effluents A and B reported in Chapter 6, Section 6.3.1 (Annex A-2).

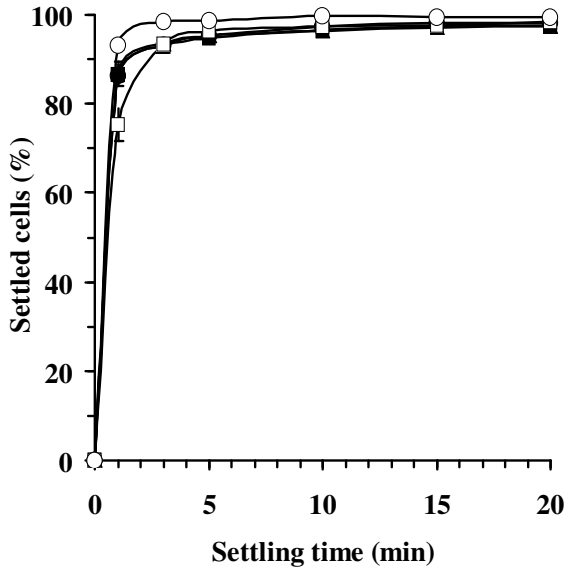

Figure 2.3. Time course of sedimentation of *Saccharomyces cerevisiae* NCYC 1364 in the presence of different cations. 5 g dry weight/l was suspended in MES pH buffer (10 mM, pH 6.0), containing 0.2 mM of each cation: (■) Cu^{2+} + Ni^{2+}; (●) Cu^{2+} + Zn^{2+}; (▲) Cu^{2+} + Cd^{2+}; (□)Cu^{2+} + Cr^{3+}; (○) Ca^{2+} (control ion). Values are the mean of three replicates of two independent experiments; standard deviations are presented (n =6).

These results are in agreement with the data described in the literature in which flocculent cells are able to flocculate in a single solution containing Zn^{2+}, Cu^{2+} Cd^{2+} or Ni^{2+} (Soares *et al.* 2002). Among the most common heavy metals, Pb^{2+} inhibits competitively yeast flocculation; however, the presence of a diminute amount of calcium in an effluent is capable of reverting the Pb^{2+} inhibition (Gouveia and Soares 2004).

Together, these results show that flocculation can be used as a cell separation process in effluents containing heavy metals. This fact allows the use of different configurations of suspended biomass reactors operating in batch or continuous mode. For example in a stirred batch tank reactor, small flocs are produced due to the intense mixing and the available surface area increases. Additionally, the stirring ensures a good homogeneity and mass transfer between the liquid and solid phases. After the contact time to remove the metals, sedimentation of biomass was achieved in few minutes. This process allowed an effective cell separation and recovery. Thus, operational and maintenance costs are minimized since no additional power source for solid-liquid separation step is needed.

2.3.3. *Effect of biomass concentration*

In order to optimize the conditions of yeast cell separation, different amounts of biomass were tested. For 2 x 10^6 cells/ml (which corresponds to 0.1 g dry weight biomass/l) no sedimentation was observed (data not shown); for 0.25 g dry weight biomass/l, a poor sedimentation occurred, while for a cell concentration above 0.5 g dry weight biomass/l, a full sedimentation was found (Figure 2.4).

This means that the presence of a minimum cell concentration above which flocculation process starts is necessary; this observation is in agreement with the concept of "critical cell density" proposed by Miki et al. (Miki *et al.* 1982a; 1982b). According to this concept, the presence of a low number of cells/ml limits the physical possibility of establishing a flocculent bond and the rate of aggregation is practically zero. In the case of the strain used in this work, a cell concentration above 0.5 g dry weight biomass/l should be used in order to obtain an efficient solid-liquid separation step.

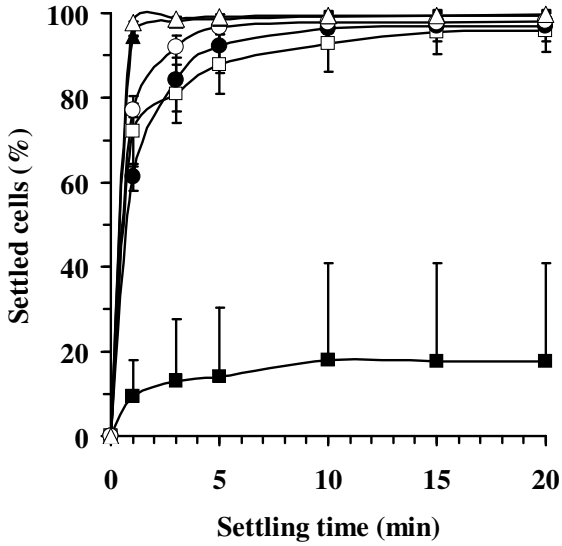

Figure 2.4. Effect of the initial biomass concentration in the settling profile of *Saccharomyces cerevisiae* NCYC 1364. (■) 0.25, (□) 0.5, (●) 1.0, (○) 2.0, (▲) 5.0 and (Δ) 10.0 g dry weight biomass/l, were suspended in 10 mM MES buffer, at pH 6.0, with 4 mM Ca^{2+}. Values are the mean of three replicates of two independent experiments; standard deviations are presented (n =6).

2.3.4. *Effect of killing temperature*

Yeast flocculation, is a surface phenomenon, which occurs in live and dead cells (Stratford 1992). On the other hand, heat treatment is one of the most common and best techniques used to produce dead biomass (Tsezos 1990). Thus, the effect of the killing temperature on yeast sedimentation properties was studied. Yeast cells killed at 80 ºC showed a decrease of 40% of the sedimentation ability (Figure 2.5); on the other hand, yeasts heat treated at 45 or 55 ºC retained their sedimentation ability (Figure 2.5).

Our results are in agreement with the literature, where it is reported a "melting" of flocs around 50-60 ºC, which is readily reversible on cooling (Mill 1964; Taylor and Orton 1975). The irreversible reducing of sedimentation profile for yeasts killed

at 80ºC observed in the present work can be most likely attributed to an irreversible denaturation of yeast flocculation lectins.

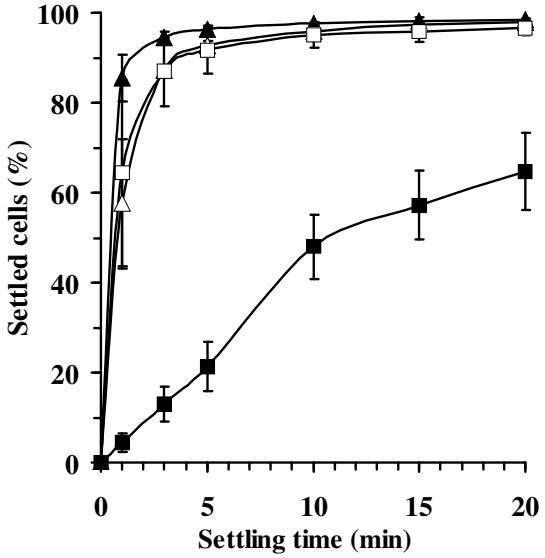

Figure 2.5. Effect of killing temperature in yeast cell separation. Yeast biomass (5 g dry weight/l) of *Saccharomyces cerevisiae* NCYC 1364 was suspended in 10 mM MES buffer, at pH 6.0, with 4 mM Ca^{2+}: (□) living cells (control) or cells heat treated at (▲) 45 ºC, (Δ) 55 ºC or (■) 80 ºC. Values are the mean of three replicates of two independent experiments; standard deviations are presented (n =6).

Yeast cells heat treated at 45 ºC and washed three times with 30 mM EDTA (in order to remove the bivalent cations adsorbed to cell wall) and twice with deionised water shows a full sedimentation ability when resuspended in the effluent described in Section 2.3.5, as well as in the effluent reported in Chapter 4, Section 4.3.1 and in the effluents A and B reported in Chapter 6, Section 6.3.1 (Annex A-3). These results strongly reinforce the previous idea of Soares *et al.* (Soares *et al.* 2002), that heavy metals can occupy lectin Ca^{2+} binding sites, inducing the correct conformation of the lectins.

The increase of killing temperature induces morphological alterations, which were more pronounced at 80ºC (Figure 2.6), with a decrease of yeast cell volume from 115 ± 8 μm^3 in live cells to 53 ± 3 μm^3 in cells killed at 80 ºC (Figure 2.7).

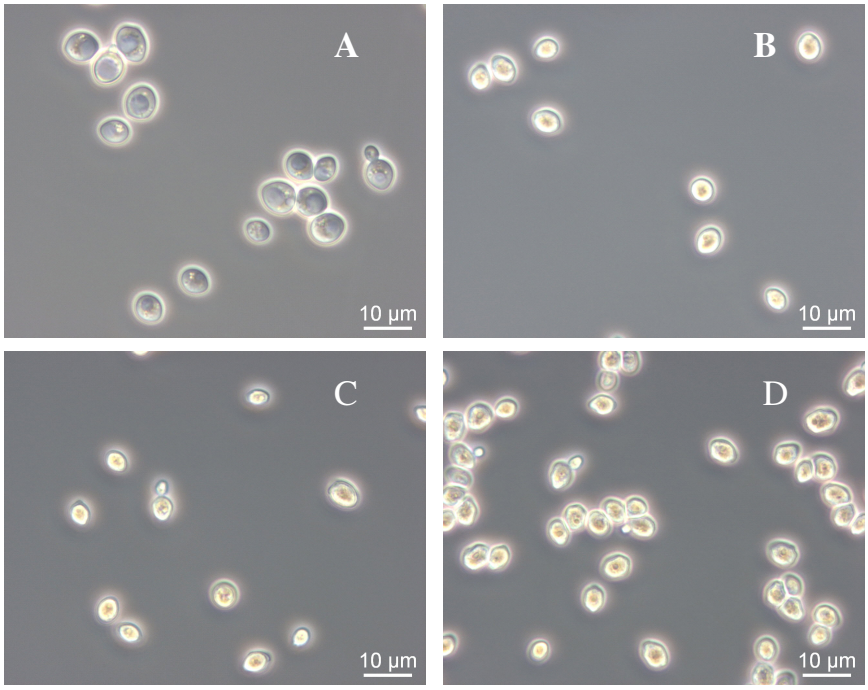

Figure 2.6. Photomicrograph of phase-contrast observation of the effect of killing temperature on yeast cell morphology of *Saccharomyces cerevisiae* NCYC 1364. A – live cells; B, C and D – cells heat treated at 45, 55 and 80 ºC, respectively.

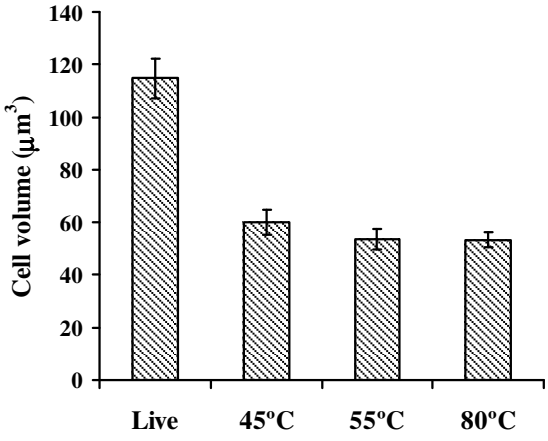

Figure 2.7. Effect of killing temperature on the mean cell volume of *Saccharomyces cerevisiae* NCYC 1364. Values are obtained from three independent experiments. In each experiment, at least 300 cells of each type (live or heat treated at 45, 55 and 80 ºC) were measured.

Cells with injured plasma membranes (non-viable cells) incorporates PI, which stains double-stranded nucleic acids (Haugland 2005). All cell population killed at 45 ºC incorporates PI (see Chapter 3, Section 3.3.3, Figure 3.4). For cells killed at 55 ºC and 80 ºC it is possible to observe cells not stained by PI (Figure 2.8).

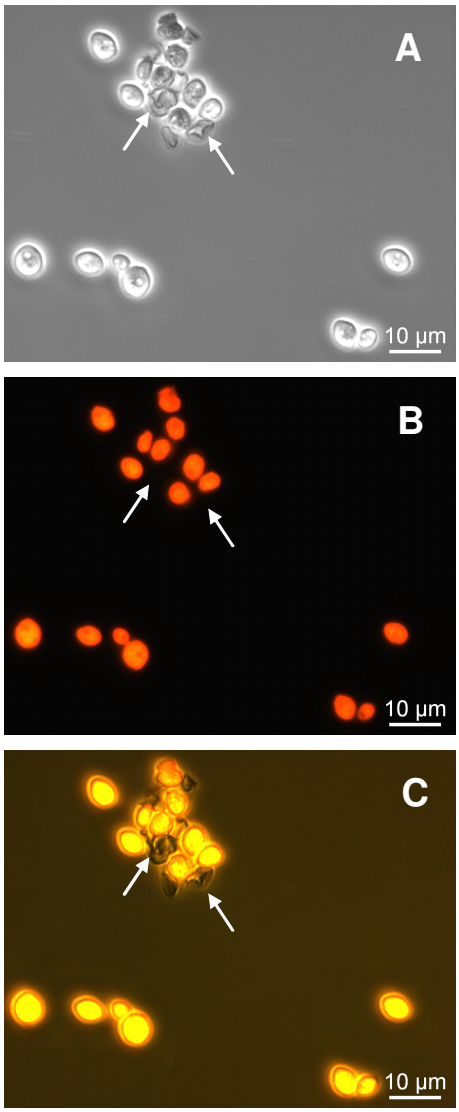

Figure 2.8. Photomicrograph of the presence of ghost cells of *Saccharomyces cerevisiae* NCYC 1364, heat treated at 80 ºC. Ghost cells are indicated by the arrows. Phase-contrast observation (A), Fluorescence microscopy (B) and Fluorescence microscopy plus Phase-contrast (C) of the same cells.

These non stained cells represent most likely ghost cells, i.e., cells which intracellular content was lost, probably due to a complete disruption of the yeast cell envelop (Figure 2.8). Together, these results demonstrate a deep modification in the yeast cell morphology, flocculation properties and cell structure of the heat treated cells at 55 ºC or 80 ºC. This fact leaded us to use a killing temperature of 45 ºC. This temperature corresponds to the smallest modification of the yeast cell morphology and for which no modification of the sedimentation profile occurred.

2.3.5. *Bioremediation of a synthetic effluent using a batch reaction system*

The feasibility of using yeast cells inactivated at 45 ºC in the removal of heavy metals from a solution was tested using a synthetic effluent, which composition simulates an electroplating plant, containing Ni^{2+}, Cu^{2+} and Cr^{3+}. This effluent contains nickel ions in excess of the stipulated criteria for both drinking water and discharge in a river (Table 2.1).

Table 2.1. Bioremediation of a synthetic waste water using a batch reaction system.

	Metal concentration (mg/l)#					
	Drinking water	**Limit discharge of effluent**	**Original effluent**	**After 1st batch**	**After 2nd batch**	**After 3rd batch**
Ni	0.02	2.0	22 ± 2	4.8 ± 0.1	1.90 ± 0.09	0.98 ± 0.09
Cu	0.002	1.0	0.27 ± 0.02	ND	ND	ND
Cr	0.05	2.0	< 0.6	-	-	-

The level of the metals reached are compared to the stipulated European Community drinking water criteria (European Union, Council Directive 98/83 EC) and Portuguese wastewater limit discharge criteria (Decreto lei 236/98).
#Mean and standard deviation of two independent experiments performed in duplicate *(n=4)*. ND: not determined; the metal concentrations were below the legal limit of discharge.

In the first batch, the percentage of Ni^{2+} removal was 78% (Figure 2.9); in the second batch, the replacement with new biomass lowered the amount of Ni^{2+} in

solution to 1.9 mg/l (Table 2.1), which meets the quality criteria for industrial effluents discharge (2 mg/l), according to the Portuguese law (Decreto-lei 236/98). After third batch, the concentration of Ni^{2+} lowered to half of the concentration obtained in the second batch (Table 2.1) being 96% of the total Ni^{2+} removed (Figure 2.9). Thus, a serial batch reactor proved to be an efficient process in the bioremediation of the effluent.

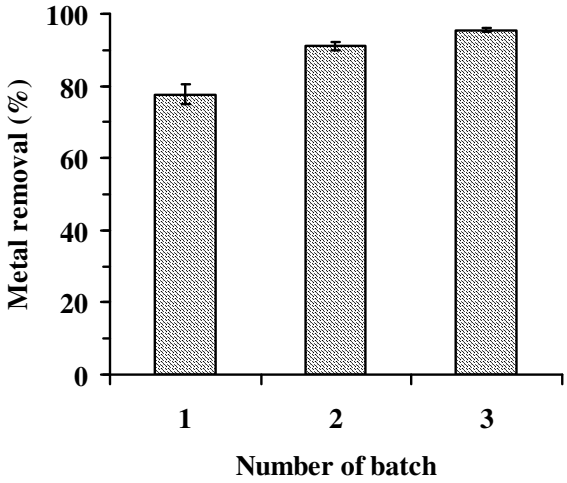

Figure 2.9. Evolution of the % of nickel removal by *Saccharomyces cerevisiae* NCYC 1364 through a series of sequential batches. 12 g dry weight/l of biomass were added to a synthetic effluent containing 22 mg/l Ni^{2+}, in 500 ml plastic flasks and shaken at 150 rpm, at 25ºC. After 30 minutes of contact of the biomass with the effluent, cells were removed and the supernatant added to fresh yeast biomass, in a subsequent batch. Values are the mean of two replicates of two independent experiments; standard deviations are presented (n =4).

2.4. Conclusions

Flocculation can be used as a fast, easy and cheap method of cell separation in a wide range of industrial effluents containing Cu, Ni, Zn, Cd and Cr facilitating further recovery and recycling of the biomass. This process consistently achieved more than 95% recovery of biomass after 5 minutes. The amount of cells in solution is a key factor; in the case of the strain NCYC 1364, used in this work, a cell

concentration $\geq$ to 0.5 g dry weight biomass/l is necessary for obtaining an efficient solid-liquid separation.

Flocculation is a surface phenomenon, which can be used as separation process of live and dead cells. A temperature of 45 ºC was the most appropriate to inactivate yeast cells since no loss of sedimentation occurred and morphologic and cytological changes were minimized.

Flocculent cells were able to remove heavy metals from a synthetic effluent. The application of a serial batch reactor allowed, after the second batch, to reduce the amount of metal in solution below to the limit of discharge of wastewater in natural waters; this corresponds to a total removal of 91% of Ni^{2+}. A subsequent batch leads to a reduction of 96% of Ni^{2+} in solution.

Moreover, based on the natural aggregation properties of a brewer's yeast, different configurations of suspended biomass reactor can be considered without the risk of biomass washout. These systems can be feasibly engineered on a large scale, requiring a low operational and maintenance costs. In addition, the cell separation process does not require an energy input.

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Chapter 3

Removal of heavy metals using a brewer's yeast strain of *Saccharomyces cerevisiae*: advantages of using dead biomass[2]

Abstract

The capacity of live and heat-killed cells at 45 ºC of *Saccharomyces cerevisiae* for the removal of Cu^{2+}, Ni^{2+} and Zn^{2+} from solution was compared. Kinetic studies have shown a maximum accumulation of Ni^{2+} and Zn^{2+} after 10 minutes for both types of cells, while for Cu^{2+}, this was attained after 30 and 60 minutes for dead and live cells, respectively. Equilibrium studies have shown that inactivated biomass displayed a greater Zn^{2+} and Ni^{2+} accumulation than live yeasts. For Cu^{2+}, live and dead cells showed similar accumulation. Fluorescence, scanning electron microscopy and infrared spectroscopy studies have shown that no appreciable structural or molecular changes occurred in the cells during the killing process. The increased metal uptake observed in dead cells can be most likely explained by the loss of membrane integrity, which allows the exposition of further metal-binding sites present inside the cells. Heat-killed cells showed higher heavy metals removal than live cells; these results point out that heat killed cells at 45 ºC are more suitable for further bioremediation works. Dead flocculent cells can be used in a low cost technology for detoxifying metal-bearing effluents since it combines an efficient metal removal with the ease of cell separation.

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3.1. Introduction

Heavy metals pollution is a growing problem mainly caused by industrialization which concerns particularly developed countries. Electroplating, surface finishing, metallurgy, mining, mineral and electronic processing are examples of industries that produce large quantities of wastewaters containing high concentrations of heavy metals. The problem of heavy metal pollution is basically associated with: a) acute toxicity linked with particular metals (Cu, Hg or Cd), even in lower concentration; b) the fact that heavy metals are not degraded or destroyed and tend to circulate; thus, they remain for a long time in nature and are accumulated through the food chain, which creates a threat to public health.

Metal-bearing effluents require a pre-treatment step before being discharged in a water body or directed to a municipal sewage treatment plant. Conventional technologies, namely chemical and physical treatments, which include precipitation-filtration, ion exchange and membrane technologies have been proposed to remove heavy metals from wastewaters. For large volumes of wastewater containing relative low metal concentrations, these technologies are not adequate (the metal removal is incomplete) or often economically prohibitive and/or impracticable. In the case of precipitation, the technology most widely used, toxic sludges are generated. Thus, the problem of metallic pollution of these hazardous sludges remains due to the difficulties of disposal (Volesky 2003).

Biological processes are an alternative to the traditional treatments. It has been reported that algae, bacteria, filamentous fungi and yeast have the ability to remove heavy metals from solutions (Volesky and Holan 1995; Bakkaloglu *et al.* 1998; Vieira and Volesky 2000; Wang and Chen 2006). The application of microbial biomass presents several advantages such as a low operating cost, minimization of the volume of chemical and/or biological sludges produced and high efficiency in detoxifying dilute effluents (Volesky 2001). Among the different kinds of biomass available, yeast

cells of *S. cerevisiae* constitute a good alternative wastewater treatment (Ferraz and Teixeira 1999; Kim *et al.* 2005; Wang and Chen 2006).

The use of live or dead cells for heavy metals removal from wastewaters is a debatable question. Live biomass, especially yeast cells, may have a higher metal accumulating capacities than dead cells due to the transport of metals across the cell membrane (Norris and Kelly 1977; Avery and Tobin 1992; Volesky *et al.* 1993). On the other hand, some authors have emphasised the advantages of using dead biomass for industrial applications: ease of storage, insensitiveness to metal toxicity and the fact that no nutrient supply is required (Gadd 1990; Volesky 1990; Gadd 1993). In order to enhance metal uptake capacities, different techniques for killing and processing biomass, such as the treatment of *S. cerevisiae* cells with alkaline solutions, detergents, ethanol, heat drying or the modification of surface biomass with cetyl trimethyl ammonium, were developed (Gadd 1990; Avery and Tobin 1992; Lu and Wilkins 1996; Ashkenazy *et al.* 1997; Bingol *et al.* 2004; Göksungur *et al.* 2005). However, it was reported that hot alkali yeast treatment can moderately decrease Cu^{2+} accumulation, when compared with native (untreated) yeast cells (Brady *et al.* 1994a); similarly, other authors reported that dead biomass shows a lower Sr^{2+} (Avery and Tobin 1992) or Pb^{2+} uptake (Suh and Kim 2000) than the respective live cells.

In this work, a detailed comparison (using kinetic and equilibrium uptake studies) of the ability of live and dead (killed at 45 ºC) flocculent cells of *Saccharomyces cerevisiae* to accumulate nickel, copper and zinc was carried out. To our knowledge, this is the first work that compares, under identical conditions, the accumulation of different metals by live and cells killed at mild temperature. Furthermore, possible structural and molecular modifications of dead biomass were examined in order to elucidate the different accumulation ability of dead and live biomass. Finally, the advantages of using flocculent heat-killed biomass in the development of a clean technology for heavy metals removal from industrial effluents are discussed.

3.2. Materials and methods

3.2.1. *Strains, media and culture conditions*

In this work, the flocculent brewing strain of *Saccharomyces cerevisiae* National Collection of Yeast Culture (NCYC) 1364 was used.

Additional information about culture maintenance, pre-cultures and cultures was described previously (Chapter 2, Section 2.2.1).

3.2.2. *Preparation of cell suspensions*

Cell suspensions were prepared as describe in Chapter 2 (Section 2.2.2.). Dead cells were obtained by drying live biomass at 45 ºC until constant weight.

3.2.3. *Determination of biomass*

Cell concentration was determined spectrophotometrically (Unicam, Helios γ) at 600 nm after appropriate dilution of the samples with EDTA solution (30 mM) to prevent cell aggregation. Calibration curves (absorbance *versus* either number of cells or dry weight) were previously made.

3.2.4. *Metals accumulation experiments*

3.2.4.1. Kinetic studies

Kinetic studies were performed in 500 ml plastic flasks containing 100 ml of cell suspension (4 g/l dry weight biomass) in MES pH buffer (10 mM at pH 6.0). Metals (Cu, Ni or Zn) were added in a final concentration of 5 or 100 mg/l from a 2000 mg/l [Cu $(NO_3)_2$ and Zn Cl_2] or 1000 mg/l [$NiCl_2$] metal stock solution $NiCl_2$ (Merck). Cell suspensions were agitated at 150 rpm, in an orbital shaker (Braun Certomat S, Melsungen, Germany), at 25 ºC. At defined intervals of time, samples (10 ml) were taken and filtered through a 0.45 μm-pore size filter. Control experiments in the absence of biomass and in the same experimental conditions described above were performed; these controls showed that no appreciable amount of metals (less than 1%) was adsorbed to the plastic flasks and to the filter membrane. At the end of kinetic studies, no bacterial contamination, checked by phase-contrast microscopy, was observed in the yeast cell suspensions.

Heavy metals were determined in the filtrates by AAS-FA in a Perkin Elmer AAnalyst 400 spectrometer (Norwalk, CT, USA), after appropriate dilution of the samples.

The amount of metal uptake, q (μmol of metal/g dry weight biomass), at each reaction time was calculated using the following equation:

$$q = V \text{ x } (C_i - C_t) \text{ x } 1000/(m \text{ x } M) \qquad [3\text{-}1]$$

where, V is the volume of reaction mixture (l), C_i and C_t are the metal concentrations in solution (mg/l) at initial and defined periods of time indicated in the figures, respectively; m is the amount of added dry weight biomass (g) and M is the molar mass of the metal.

3.2.4.2. Equilibrium studies

Equilibrium metal studies were carried out in 100 ml plastic flasks containing 30 ml of cell suspension (4 g/l dry weight biomass) in MES pH buffer (10 mM at pH 6.0) with an appropriate concentration of metal (between 5 to 200 mg/l for nickel and from 5 to 50 mg/l for copper and zinc). Cell suspensions were shaken at 150 rpm at 25 ºC. After 1 hour of contact of biomass with metals, samples were collected and filtered through a 0.45 μm-pore size filter. Filtrates were analysed for metals equilibrium concentration by AAS.

3.2.4.3. Equilibrium model

The Langmuir adsorption model, described in Chapter 1 (Section 1.2.5), was used to evaluate the metals uptake of live and dead biomass at 45 ºC:

$$q = q_{max}\, b\, C_{eq} / (1 + b\, C_{eq}) \qquad [3\text{-}2]$$

where, q is the metal specific uptake (μmol of metal/g dry weight biomass), q_{max} (μmol of metal/g dry weight biomass) is the maximum metal uptake biomass capacity, b is the Langmuir constant (l/μmol of metal); C_{eq} is the metal equilibrium concentration (μmol/l).

3.2.5. *Evaluation of lethality induced by heat and* Cu^{2+}

Cells heated at 45 ºC until constant weight in sterile conditions were suspended in deionised water at $1x10^8$ cells/ml. Four replicates of 100 μl of cell suspension were incubated at 25 ºC during one week.

In the case of lethality induced by Cu^{2+}, cells were suspended in 10 mM MES pH buffer (pH 6.0) in a final concentration of 4 g dry weight biomass/l; i.e., the same conditions used in the kinetic studies. Before and after the addition of 5 mg/l of metal, samples (two replicates of 100 µl) were taken at defined intervals of time, serially diluted with sterile deionised water and plated on YEPD agar (two replicates of 100-200 µl of convenient dilutions). After 3-4 days of incubation at 25 ºC, colonies were counted; no further colonies were observed when incubation was prolonged during one week.

3.2.6. *Assessing of membrane integrity*

Dead cells were stained with Propidium Iodide and observed in an epifluorescence microscope; images were acquired and processed as described in Chapter 2 (Section 2.2.4).

3.2.7. *Staining of cell walls*

Live and dead cells, at $1x10^7$cells/ml, were washed twice and resuspended in water. Calcofluor white M2R (Sigma, Steinheim, Germany) was added to a final concentration of 25 µM. Cell suspensions were incubated in the dark at room temperature during 30 minutes. Then, cells were washed twice with deionised water and examined using an epifluorescence microscope equipped with a HBO-100 mercury lamp and a filter set A (excitation filter BP 340-380, dichromatic mirror 400 and suppression filter LP 425) from Leica. Images were acquired and processed as described in Chapter 2 (Section 2.2.4).

3.2.8. *Scanning electron microscopy*

Cells were fixed by glutaraldehyde 3% (w/v) (Fluka) during 2 hours with an agitation of 100 rpm at room temperature. Then, cells were centrifuged (500 *g*, 5 minutes), washed three times with 0.1 M of sodium cacodylate buffer (pH 6.8) (Sigma) and resuspended in osmium tetroxide (2%) (Fluka). After 1 hour of incubation at room temperature with an agitation of 100 rpm, cells were washed with 0.1 M of sodium cacodylate buffer (pH 6.8), centrifuged and resuspended in deionised water.

Cell suspensions were transferred to a microscope slide previously coated with 0.1% (w/v) of aqueous solution of poly-L-lysine (Sigma). Fixed cells were dehydrated by incubation in a series of ethanol concentration [10, 20, 30, 50, 70 and 100% (v/v)] during 10 minutes for each sample. Next, cells were dried by critical point using liquid dioxide carbon as transition solvent in a Balzer's apparatus. Then, samples were coated with gold using a SEM coating unit (Polaron SC 500) and examined in a JEOL JSM-35C scanning electron microscope at 15 kV and x 10000 magnification.

3.2.9. *Infrared spectrums*

Infrared spectrums of live and dead cells (heated at 45 ºC), as well as dead biomass exposed to copper, nickel or zinc, were performed. Live or dead biomass (2 g/l dry weight biomass) was washed as described in the item preparation of cells suspension. In the case of dead biomass, this was suspended in MES pH buffer (10 mM at pH 6.0) containing solutions of metals at concentrations of 22.2 mg/l Cu, 32.3 mg/l Ni or 26.2 mg/l Zn in order to achieve the saturation of biomass by the different metals. Cell suspensions were agitated at 150 rpm during 1 hour, at 25 ºC, in plastic flasks and then dried at 25 ºC before being analysed in an infrared spectrometer with Fourier transform (FTIR) (Bomem MB- 154 S). Disks of 100 mg

of KBr containing 3% (w/w) of finely ground power of each sample were prepared and then examined. Spectra were measured between 4000 and 650 cm^{-1} and further treated with Win-Bomem Easy v3.04 level software.

3.2.10. *Reproducibility of the studies*

All experiments were repeated independently at least two times. In kinetic and equilibrium studies, each repetition was carried out in duplicate.

3.3. Results

3.3.1. *Kinetic studies*

The kinetics of metals accumulation (Cu^{2+}, Ni^{2+} and Zn^{2+}) by live and dead cells of *S. cerevisiae* were performed in buffer solution, at pH 6.0, using two different concentrations: 5 mg/l and 100 mg/l. These metals concentrations were selected because they correspond to a lower (5 mg/l) and a higher (100 mg/l) metal concentration found in the electroplating wastewater streams. Copper kinetics studies were only performed for the lowest concentration due to early precipitation at pH 6 (Hogfeldt 1982). Kinetic studies are important both in an academic and applied point of views, since the information is useful for determining the contact time required for equilibrium uptake studies and for removal of the metals from the effluents.

For an initial copper concentration of 5 mg/l, after 15 minutes of contact time of live or dead biomass, 38 and 44% of metal was removed. This represents an accumulation of approximately 7.0 and 8.3 μmol/g dry weight biomass, respectively (Figure 3.1-A). Increasing the contact time for 30 minutes, resulted in a slight increase of metal removal: 41 and 50% of copper was removed by live and dead cells,

which represents an accumulation of approximately 7.6 and 9.6 Cu^{2+} μmol/g dry weight biomass, respectively. For dead cells, equilibrium was attained after 30 minutes while with live cells equilibrium was only observed after 60 minutes where an accumulation of approximately 9.1 μmol/g dry weight biomass was obtained, which represents 49% of Cu^{2+} removal (Figure 3.1-A). Extending the incubation time between biomass (live or dead) and metals up to 24 hours, no increase of metal uptake was observed.

For live and dead biomass, nickel and zinc kinetics accumulation were faster than with copper. For both nickel concentrations tested, the maximum nickel uptake occurred after 10 minutes of contact time (Figures 3.1-B and 3.2-A). For 5 mg/l nickel concentration, live and killed cells accumulated 3.7 and 6.4 Ni^{2+} μmol/g dry weight biomass, respectively (Figure 3.1-B); for 100 mg/l nickel concentration, live and heat-killed cells accumulated 24 and 84 Ni^{2+} μmol/gdry weight biomass, respectively (Figure 3.2-A). For the lower zinc concentration (5 mg/l), the same behaviour was observed: live or heat-killed biomass accumulated 3.8 and 11 μmol/g dry weight biomass, respectively, after 10 minutes of contact time (Figure 3.1-C). For the highest zinc concentration (100 mg/l), dead cells presented a slight increase of metal removal after one and three hours of contact time [44 and 48%, which corresponds to approximately 142 and 160 Zn^{2+} μmol/g dry weight biomass, respectively (Figure 3.2-B)] when compared with the value obtained after 10 minutes of contact time [40%, which corresponds to approximately 128 Zn^{2+} μmol g^{-1}dry weight biomass (Figure 3.2-B)].

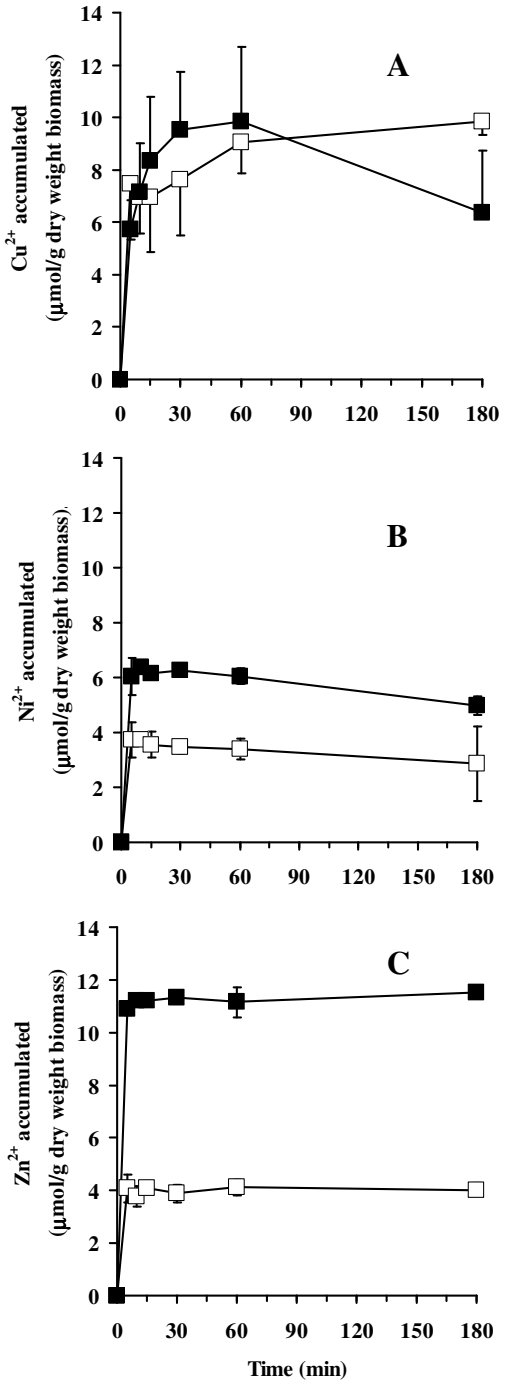

Figure 3.1. Accumulation of Cu^{2+} (A), Ni^{2+} (B) and Zn^{2+} (C) by live (□) or dead (■) cells of *Saccharomyces cerevisiae* NCYC 1364. Cells were suspended in 10 mM MES buffer at pH 6.0 in a final concentration of 4 g/l dry weight biomass; the initial metal concentration was 5 mg/l. Each point represents the mean of at least two independent experiments performed in duplicate; standard deviations are presented ($n \geq 4$).

Similarly to what happened with copper, the extension of incubation time up to 24 hours resulted in no increase nickel or zinc removal by live or dead biomass for either metal concentrations tested.

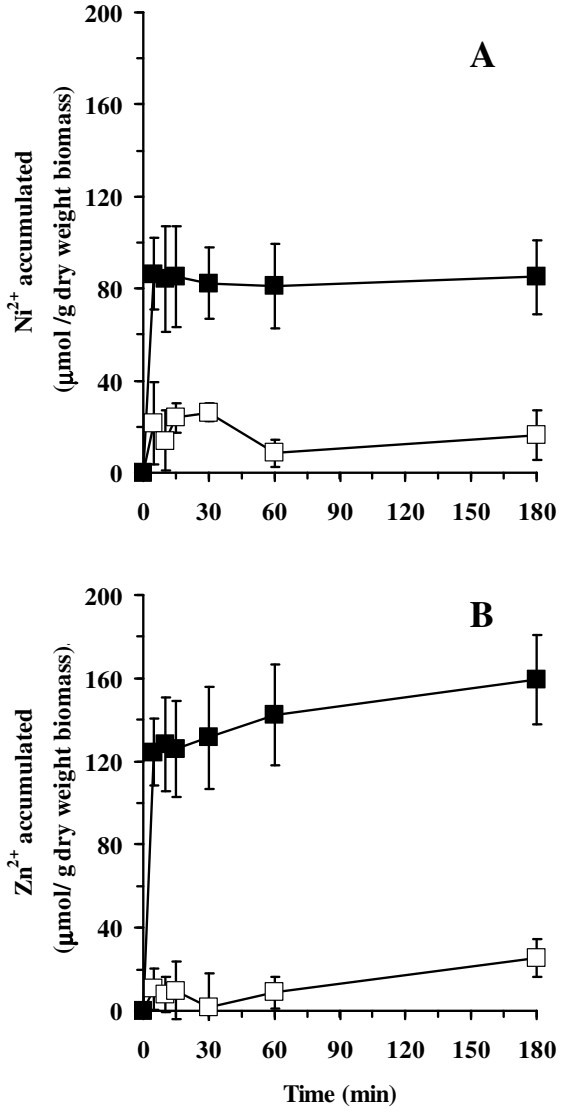

Figure 3.2. Accumulation of Ni^{2+} (A) and Zn^{2+} (B) by live (□) or dead (■) cells of *Saccharomyces cerevisiae* NCYC 1364. Cells were suspended in 10 mM MES buffer at pH 6.0 in a final concentration of 4 g/l dry weight biomass; the initial metal concentration was 100 mg/l. Each point represents the mean of at least two independent experiments performed in duplicate; standard deviations are presented ($n \geq 4$).

3.3.2. *Equilibrium studies*

For the objective of comparing metals (Cu^{2+}, Ni^{2+} and Zn^{2+}) uptake performance by live and heat-treated cells of ale brewing flocculent strain of *S. cerevisiae* NCYC 1364, equilibrium experiments were carried out at fixed pH (6.0) and temperature (25 ºC). As it was previously established in kinetic studies reported above (Figures 3.1 and 3.2), a contact time of 60 minutes was used to ensure that equilibrium between live or dead cells and all three metals was attained.

Metals uptakes (*q*) by the two types of biomass were plotted against the equilibrium metal concentration (*Ceq*) present in solution (adsorption isotherms) (Figure 3.3). For live and dead biomass, Ni^{2+} or Zn^{2+} uptakes increased with metal concentrations until biomass reached saturation (Figure 3.3-B and 3.3-C). Heat-inactivated biomass displayed a greater Ni^{2+} and Zn^{2+} accumulation than live yeasts (Figure 3.3-B and 3.3-C). In the case of Cu^{2+}, a biphasic behaviour seems to occur for both types of biomass studied; however, no apparent saturation of biomass binding sites seems to be achieved (Figure 3.3-A).

In order to characterize metals uptake by the two types of yeast biomass, Langmuir model was applied to the experimental data. Unless for live biomass in the presence of Zn^{2+}, for which a poor correlation was found (r = 0.854), the Langmuir model fitted quite well into the experimental data (Table 3.1). The comparative analysis of q_{max} values (this parameter provides information about the maximum metal accumulation capacity) showed that heat inactivated biomass accumulated more Ni^{2+} and Zn^{2+} (about 17 and 14 more times, respectively) than live biomass (Table 3.1).

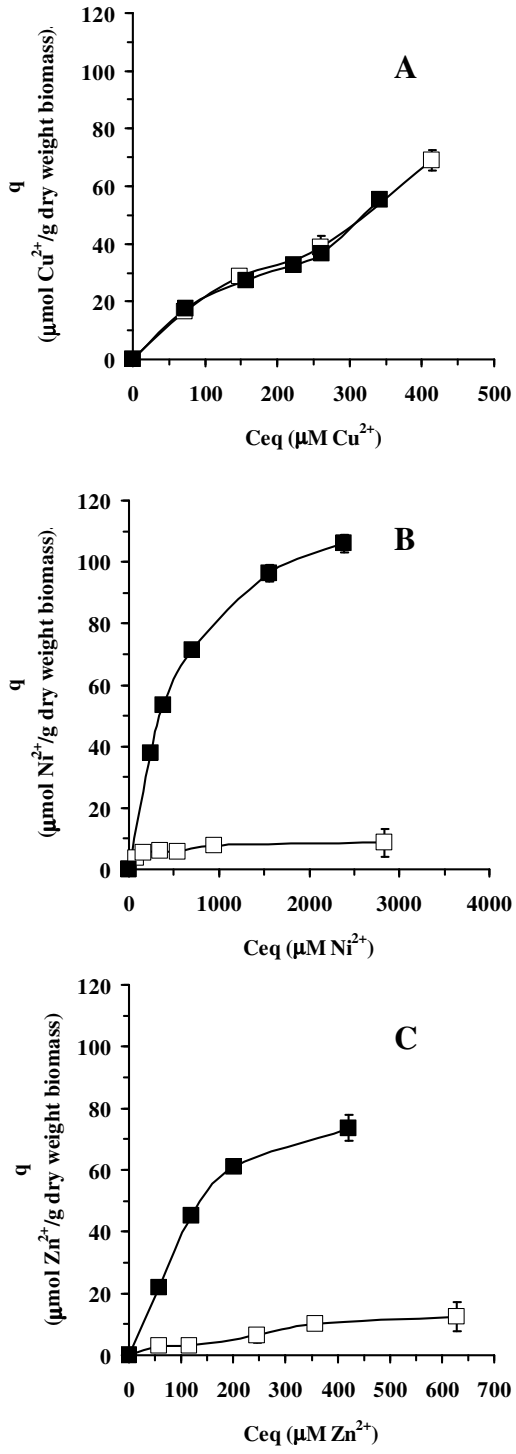

Figure 3.3. Cu^{2+} (A), Ni^{2+} (B) and Zn^{2+} (C) isotherms for live (□) or dead (■) cells of *S. cerevisiae* NCYC 1364. Cells were suspended in 10 mM MES buffer at pH 6.0 in a final concentration of 4 g/l dry weight biomass. The results were obtained after 1hour of contact time between biomass and metals. Each point represents the mean of at least two independent experiments performed in duplicate; standard deviations are presented ($n \geq 4$).

For copper, live and dead biomass displayed similar accumulation capacities: 122 μmol Cu^{2+}/g dry weight biomass and 156 μmol Cu^{2+}/g dry weight biomass, respectively (Table 3.1). For live biomass, metal uptake ability decreased in the following order: Cu^{2+} >> Zn^{2+} and Ni^{2+}; for dead biomass, a similar accumulation pattern was found.

Table 3.1. Langmuir parameters for Cu^{2+}, Ni^{2+} and Zn^{2+} removal.

Metal	q_{max} (μmol/g dry weight biomass)		b (l/μmol metal)		r	
	Live	**Dead**	**Live**	**Dead**	**Live**	**Dead**
Cu^{2+}	122	156	2.1	1.3	0.992	0.945
Ni^{2+}	8	134	11	1.7	0.957	0.999
Zn^{2+}	12	163	5	2.8	0.854	0.990

3.3.3. *Accessing of structural and molecular modifications of dead biomass*

For the purpose of explaining modifications of metals uptake by dead biomass when compared with live cells, different studies for assessing structural and molecular modifications of heat-treated biomass were conducted.

Membrane integrity was assessed using propidium iodide (PI). PI is a membrane impermeant stain and is generally excluded from viable cells. Cells with damaged plasma membrane incorporate PI; this stain binds to DNA by intercalating between the bases with little or no sequence and exhibits an orange-red fluorescence (Haugland 2005). As it can be seen in Figure 3.4, all cell population heat-treated at 45 ºC incorporates PI as a consequence of membrane integrity lost during the thermal treatment. This killing effect was additionally confirmed by a cultural procedure: cell suspension dried at 45 ºC was incubated during one week at 25 ºC in YEPD agar plates; no colonies were found, which demonstrated that all population was dead.

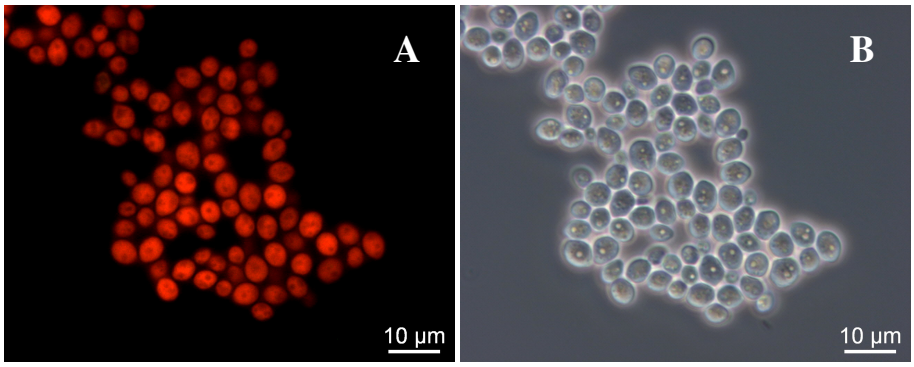

Figure 3.4. Fluorescence microscopy of cells of *S. cerevisiae* NCYC 1364 heat-killed at 45 ºC and stained with Propidium Iodide (A). Phase-contrast observation of the same cells (B).

Cell wall modifications due to thermal treatment were assessed using calcofluor white. Calcofluor white binds to chitin with high specificity (Pringle 1991). Chitin is mostly located in scars region although some of it is dispersed throughout the yeast cell wall (Cabib *et al.* 2001). Dead cells, after being stained with calcofluor white, exhibited a typical uniform faint fluorescence on their surface with well-visible bud scars (Figure 3.5). This fact suggests that no redistribution of chitin occurred during the thermal treatment.

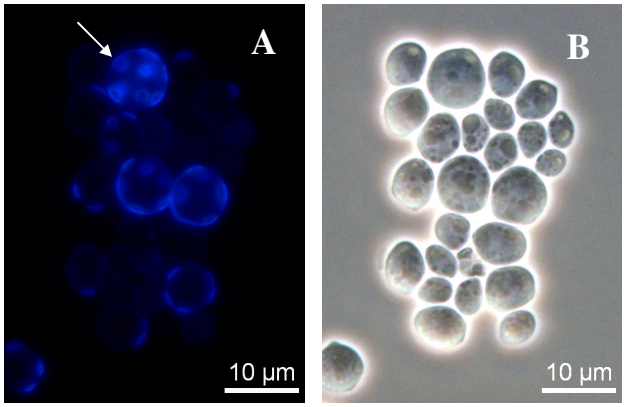

Figure 3.5. Fluorescence microscopy of *S. cerevisiae* NCYC 1364 heat-killed at 45 ºC and stained with calcofluor white M2R (A); arrow: bud car. Phase-contrast observation of the same cells (B).

Maintenance of morphological characteristics of dead cells was further confirmed by Scanning Electron Microscopy (SEM). No significant differences between live and dead cells were observed; characteristics bud scars were well-visible in dead cells (Figure 3.6).

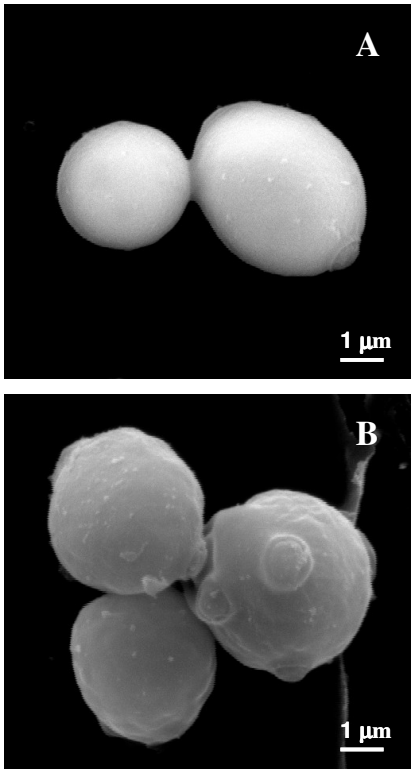

Figure 3.6. Scanning electron microscopy of *S. cerevisiae* NCYC 1364. Live (A) or heat-killed cells at 45 ºC (B). Arrow: bud car.

Molecular modifications in heat-treated biomass were investigated by FTIR spectroscopy. The spectra contain information of yeast components, which are represented by specific peaks in the fingerprinting regions: region between 790 and 1180 cm^{-1} correspond ing to the sugar component of cells; between 1200 – 1290 corresponding to nucleic acids; between 1400 and 1700 cm^{-1} representing the protein component; between 2500 – 3800 cm^{-1} corresponding to -OH and -NH groups and hydrogen bonding; finally, peaks at 2900 cm^{-1} may be due to chitin (Brugnerotto *et al.* 2001; Galichet *et al.* 2001; Padmavathy *et al.* 2003). The spectrums of live and dead

biomass were very similar and all the characteristic peaks due to the main yeast macromolecules were present in both (Figure 3.7).

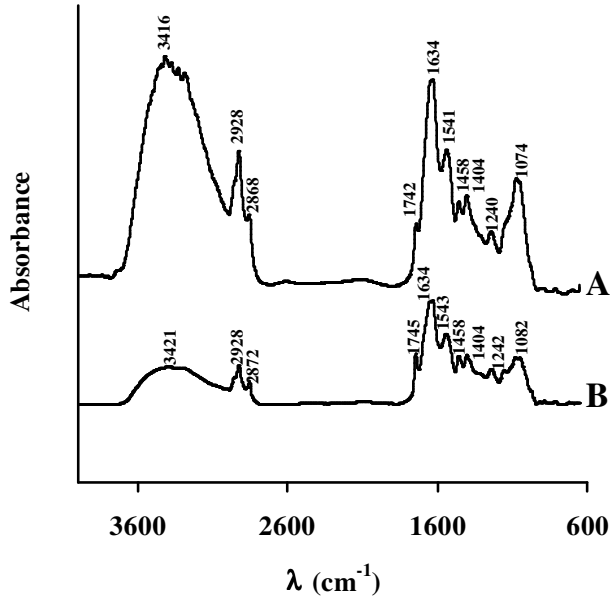

Figure 3.7. IR spectra of live and dead cells of *S. cerevisiae* NCYC 1364. Cells heated at 45 ºC (dead cells) (A); live cells (B).

More specifically, it is possible to observe a peak with a maximum at 1074 cm^{-1} (dead cells), which can be mainly attributed to the β(1→3) glucan, the major structural component of the cell wall (30-45% of wall mass). In addition, two important peaks were found in dead cells: a peak at 1541 cm^{-1} due to the presence of protein amide II band (mainly C-N stretching plus N-H bonding) and a peak at 1634 cm^{-1} due to the protein amide I band (mainly C=O stretching and contribution of N-H bonding). The peak with a maximum at 3416 cm^{-1} , recorded in dead cells, could correspond to hydroxyl groups bounded with NH groups of the secondary amide group of chitin (Padmavathy *et al.* 2003).

3.3.4. *Interaction of Cu^{2+}, Ni^{2+} and Zn^{2+} with dead biomass*

The interaction of Cu^{2+}, Ni^{2+} and Zn^{2+} with dead biomass was also investigated by FTIR technique. As it can be seen in Figure 3.8, biomass loaded with the different metals maintained yeast characteristic peaks.

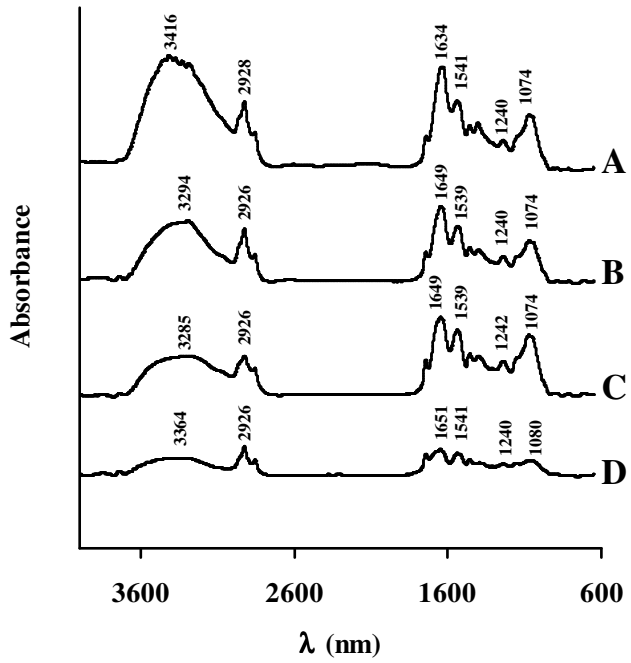

Figure 3.8. IR spectra of *S. cerevisiae* NCYC 1364 after being exposed to different heavy metals. Dead cells (control) (A); dead cells after Zn^{2+} (B), Ni^{2+} (C) and Cu^{2+} exposition (D). Dead biomass was suspended in 10 mM MES buffer at pH 6.0 in a final concentration of 2 g/l dry weight biomass. The initial metal concentration was: 22.2 Cu^{2+} mg/l, 32.3 Ni^{2+} mg/l and 26.2 Zn^{2+} mg/l. Cells were incubated in the metal solutions during 60 minutes at 25 ºC.

After metals accumulation, dead biomass showed a remarkable decrease of the band at 2500-3800 cm^{-1} (Figure 3.8) and a shift of the maximum of the peak at 3416 cm^{-1}, recorded in the absence of metals, to 3364, 3294 and 3285 cm^{-1} , in the case of biomass loaded with Cu^{2+}, Zn^{2+} and Ni^{2+}, respectively. This was probably due to the interaction of –NH and –OH groups with metals.

A small decrease of the peak at 2928 cm^{-1} was also observed with all the biomass loaded with the different metals (Figure 3.8). This was probably due to the interaction of the chitin, present in the cell wall, with metals. In addition, a decrease of the peaks in the region of proteins, 1634 and 1541 cm^{-1} (in the absence of metals), associated with a shift of their maximums to 1649 cm^{-1} (biomass loaded with Ni^{2+} and Zn^{2+}), 1651 cm^{-1} (biomass loaded with Cu^{2+}) and to 1539 cm^{-1} (biomass loaded with Zn^{2+}) was observed. Finally, a decrease of the peaks in the region of carbohydrates, namely at 1074 cm^{-1} (in the absence of metals), associated with a shift of the maximum to 1080 cm^{-1} (biomass loaded with Cu^{2+}) was observed. This fact was probably due to the interaction of glucans from the cell wall with the metals (Figure 3.8).

3.4. Discussion

The knowledge of metal accumulation characteristics by yeast biomass is of fundamental importance for further use in the bioremediation processes. Kinetic and equilibrium studies are usually carried out for quantitative assessment of biomass performance as well as for process design.

Yeast metal uptake usually occurs in two steps: the first step (biosorption) is fast (it occurs in the first minutes of contact with the metal), is metabolism independent of metabolism and happens in live and dead cells; the second step (bioaccumulation) is generally considered metabolism dependent (it occurs only in live cells) and is attributed to intracellular metal uptake across the cell membrane (Blackwell *et al.* 1995). Nickel and zinc accumulation by live or dead biomass of *S. cerevisiae* NCYC 1364 was very fast and occurred within 10 minutes; even though both metals are actively transported in *S. cerevisiae* (Fuhrmann and Rothstein 1968), no further accumulation was observed in live cells (Figure 3.1) and no appreciable toxic effects could be found for this metal concentration (5 mg/l) (Mowll and Gadd

1983; Soares *et al.* 2003). The absence of bioaccumulation step in live cells was probably due to the fact that yeast cells were not incubated in the presence of an external energy source, like glucose. Most likely, yeast internal energetic reserves were not sufficient to provoke a supplementary and detectable accumulation of nickel and zinc. Consistent with this hypothesis, an enhancement of Ni^{2+} and Zn^{2+} uptake by yeast cells pre-treated with glucose comparative to those washed untreated (starved) cells was described (Fuhrmann and Rothstein 1968; Stoll and Duncan 1996).

In previous work, it was found that the killing in mild conditions (45 ºC) is the most appropriate temperature to inactivate yeast cells (Machado *et al.* 2008). Under these conditions, yeast cells maintained their flocculation properties, when compared with live cells, most likely because no denaturation of the lectin-like proteins occurred at this temperature (Machado *et al.* 2008). In the present work, heavy metals removal by live and dead cells (inactivated at 45 ºC) was quantitatively assessed using equilibrium metal uptake studies. Taking into account the maximum metal uptakes (Q_{max}) determined at 25 ºC and pH 6.0, dead biomass was able to accumulate higher quantities of Ni^{2+} and Zn^{2+} (17 and 14 times higher, respectively) than live biomass (Table 3.1); for Cu^{2+}, live and dead cells showed a similar metal uptake capacity. This illustrates that biomass dead at 45 ºC is more suitable for bioremediation processes.

In order to understand why inactivated cells showed a higher metal accumulation capacity, different studies, namely at yeast cell wall and membrane level, were performed. The aim of these studies was to assess whether structural and molecular modifications of heat-treated biomass occurred. The cell wall of *S. cerevisiae* is the first cellular structure to be in contact with metal ions; cell wall presents a complex macromolecular structure with a layered organization constituted by an amorphous inner and a fibrilar outer layers. The inner layer is mainly composed by β-glucans and chitin. β-glucans are constituted by fractions of β(1→3) and β(1→6)-linked glucose residues; chitin is composed by linear chains of β(1→4)-linked to N-acetylglucosamine. Chitin is present as small amounts in the wall (1.5 - 6%)

predominantly located in the bud scars. The outer layer consists predominantly of α-mannans highly glycosylated and associated with proteins (mannoproteins), which corresponds to 30-50% of wall mass (Cabib *et al.* 2001; Lesage and Bussey 2006); these structures seems to be very important in heavy metals accumulation (Brady *et al.* 1994b). Calcofluor white has been very useful for assessing abnormal patterns of cell wall deposition (Pringle 1991). In the present study, assays with calcofluor white show that dead cells maintain their characteristics: chitin remained in yeast scars after thermal treatment (Figure 3.5). The maintainance of morphological characteristics of dead cells was further confirmed by SEM, where no significant differences between live and dead cells were observed (Figure 3.6). In addition, the IR spectra of live and dead biomass were very similar: all characteristic peaks attributed to the main yeast macromolecules of cell wall were present in both spectrums (Figure 3.7). Since heat-killed cells have shown a loss of plasma membrane integrity, when assessed by propidium iodide (Figure 3.4), the increase of nickel and zinc removal by dead cells could most likely be attributed to the exposition of further metal-binding sites present inside the cells. According to this, an increase of Cu^{2+} accumulation by *P. spinulosum* and of U accumulation by *S. cerevisiae* cells permeabilized by the action of detergents (Gadd 1990) or by the action of HCHO or $HgCl_2$ (Strandberg *et al.* 1981), respectively, was also described.

In the case of copper, live and dead cells showed similar removal. This can be explained by the high copper toxicity, which provokes lesions of yeast cell membrane (Soares *et al.* 2003). In the case of the strain of *Saccharomyces cerevisiae* used in the present work, 5 mg/l Cu^{2+} induced a lethality of 50% after 20 minutes of contact time and about 70% after 60 minutes (Annex B-1). Thus, live cells were progressively converted into dead cells and copper accumulation by the initial live cells became similar to dead cells (Figure 3.1).

Infrared analyses of dead biomass after being exposed to different metals showed a decrease of the peaks of the fingerprinting regions (Figure 3.8). This

suggests the involvement of carboxyl, amino, hydroxyl and amides groups of protein and carbohydrate fractions (most likely of mannoproteins, glucans and chitin) of the cell wall in the yeast metals uptake. These results are in agreement with those obtained by Padmavathy *et al.* (2003), who suggested that mannoproteins and glucans present on the cell wall were responsible for the sorption of Ni^{2+}. Brady and Duncan (1994) had shown that blocking amino, carboxyl or hydroxyl groups of the cell walls reduces the accumulation capacity of Cu^{2+}. These facts clearly indicated the participation of these functional groups in Cu^{2+} binding.

The comparison of metals uptake by *S. cerevisiae* NCYC 1364 with other *S. cerevisiae* strains is very difficult particularly due to the different operational conditions: biomass pre-treatment, biomass concentration, pH and contact time between biomass and metals. Table 3.2 shows metals accumulation by different types of *S. cerevisiae* biomass previously reported in the literature. The extend of copper uptakes by live or dead cells of *S. cerevisiae* NCYC 1364 determined in this work is similar to the values presented in Table 3.2. For the other two metals, accumulation capacities reported in Table 3.2 varies widely; the values of metals uptakes by dead cells of *S. cerevisiae* NCYC 1364 determined in this work presents intermediary values of accumulation when compared with these studies.

A widespread difficulty associated with the industrial use of microorganisms in different remediation technologies is linked with their small size and low density; these characteristics can limit the choice of suitable reactors and difficult cells separation from the reaction mixture after the effluent has been treated. The use of flocculent brewing yeast biomass is a natural, easy and cheap method of cell separation from the treated effluents, that overcomes the need of cell immobilisation (like gel immobilisation). Yeast flocculation also facilitates further recycling of the biomass and recovery of the metals.

Table 3.2. Comparison of metals uptake capacity by *S. cerevisiae*.

Metal	Type of *S. cerevisiae* biomass	Metal uptake capacity (µmol/g dry weight biomass)	pH	Time of contact* (h)	Reference
Cu	Live baker's yeast	200	4	16	(Volesky and May-Phillips 1995)
	Live brewer's yeast	160	4	16	(Volesky and May-Phillips 1995)
	Dead brewer's yeast	78	5.5	1	(Bakkaloglu *et al.* 1998)
	Dead biomass derived from whiskey destilary	90	**	2	(Bustard and McHale 1998)
	Live baker's yeast	100	6	24	(Al-Saraj *et al.* 1999)
Ni	Dead brewer's yeast	25	5.5	1	(Bakkaloglu *et al.* 1998)
	Live baker's yeast	136	6	24	(Al-Saraj *et al.* 1999)
	Dead biomass	789	5	24	(Özer and Özer 2003)
	Dead and deactivated protonated baker's yeast	194	6.75	24	(Padmavathy *et al.* 2003)
Zn	Dead brewer's yeast	53	6	1	(Bakkaloglu *et al.* 1998)
	Dead biomass derived from whiskey distillery	258	**	2	(Bustard and McHale 1998)
	Live baker's yeast	290	5	24	(Al-Saraj *et al.* 1999)

*Time of contact of the biomass with metals. **Not reported in the publication.

In conclusion, dead yeast cells (at 45 ºC) evidenced higher metals uptake capacities than live cells. This fact points out that heat killed biomass at 45 ºC seems to be a promissory biomass for further application in metal bioremediation processes. This increase of metal uptake capacity is most likely explained by the loss of cell membrane integrity; this occurs during the killing process and allows the exposition of further metal-binding sites present inside the cells. Kinetics studies have shown that a contact time of 60 minutes is enough to achieve the equilibrium between metals and dead biomass. Fluorescent and scanning electron microscopy studies, as well as IR spectroscopy studies, have shown that no appreciable structural or molecular changes occurred during the killing process. Infrared studies suggested the participation of carboxyl, amino, hydroxyl and amides groups in metals yeast uptake.

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Chapter 4

Removal of heavy metals using a brewer's yeast strain of *Saccharomyces cerevisiae*: chemical speciation as a tool in the prediction and improving of treatment efficiency of real electroplating effluents[3]

Abstract

In the present work, the influence of the competitive effect of inorganic ligands (carbonates, chlorides, fluorides, phosphates, nitrates and sulphates), which can be present in real multi-metal electroplating effluents, on the biosorption of chromium, copper, nickel and zinc ions by yeast cells of *Saccharomyces cerevisiae* was rationally examined. Additionally, chemical speciation studies allowed optimizing the amount of yeast biomass to be used in the treatment of effluents contaminated with nickel.

The applicability of chemical simulation studies was tested using two simulated effluents and validated using one real electroplating effluent, all containing high concentrations of nickel (about 303 μM). For nickel removal, heat-killed biomass of the brewing flocculent strain of *Saccharomyces cerevisiae* was used, in a batch mode. After the implementation of the bioremediation process (12 g dry weight/l of yeast cells), the concentration of nickel in the real effluent (34 μM) reached the quality criteria for industrial effluents discharge, after the second or third batch according to the U.S.-Environmental Protection Agency and Portuguese law, respectively. This corresponded to a removal of nickel of 89%.

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4.1. Introduction

The threat of heavy metal pollution to public health and wildlife lead to an enlarged interest in the development of effective technologies for heavy metals immobilization in a non-bioavailable form or their re-speciation into less toxic forms. The current technologies (precipitation, ion exchange, reverse osmosis or evaporation) have been shown inadequate or expensive; conversely, the use of a biological-based technology seems to be a very attractive way of metal ions removal (Volesky 2001).

Flocculent yeast cells of *Saccharomyces cerevisiae* constitute a new approach in the heavy metals bioremediation (Soares *et al.* 2002a). Recently, it was shown that brewing cells of *S. cerevisiae*, heat-killed at 45ºC, retain their flocculation ability in wastewaters with different heavy metals composition (Machado *et al.* 2008) and present a greater metals uptake capacities than live cells (Machado *et al.* 2009), being very promissory for use in the treatment of heavy metals-loaded effluents. Due to the auto-aggregation properties of this biomass, the solid-liquid separation process after effluent treatment is strongly facilitated, overcoming the need of cell immobilisation (like gel immobilisation) which, on large engineering scale, can be prohibitively expensive. Flocculent cells can be used in stirred tank reactors, operating in a batch or continuous mode without need of energy input for solid/liquid (biomass/effluent treated) separation stage (like it is required in centrifugation, filtration or flotation), prior to subsequent regeneration/metal recovery or disposal stage (Soares *et al.* 2002a; Machado *et al.* 2008). Moreover, the natural aggregation properties of flocculent yeast cells allows the use, in a batch mode, of the sedimentation tanks already available in the wastewater treatment plants of the electroplating industries without the need of any further capital investment.

Yeast metal accumulation is affected by several factors such as pH, redox potential (E_h), presence of anions, cations and soluble organic compounds. Some of

these factors can act at different levels simultaneously. For instance, pH of the solution affect the percentage of ionised groups of yeast cell wall; at low pH, the increase of protonation of yeast cell wall ligands decrease metals adsorption (Brady and Duncan 1994; Ferraz and Teixeira 1999; Zouboulis *et al.* 2001; Özer and Özer 2003; Vasudevan *et al.* 2003; Mapolelo and Torto 2004; Parvathi and Nagendran 2007). Simultaneously, pH affect metal speciation (the different physicochemical forms of the metal in solution, which together make up its total concentration) (Florence 1983) and consequently the available metal (i.e., the free and labile metal) to be sequestered by the microorganisms. On the other hand, the increase of pH can result in the precipitation of metals hydroxides, reducing metal accumulation by the biomass (Sheng *et al.* 2004). The presence of anions in solution can complex (carbonates, chlorides, fluorides, phosphates and sulphates) metals ions and thus reduce or inhibit their adsorption to *S. cerevisiae* (Hughes and Poole 1991; Avery and Tobin 1993; Gadd 1993). Heavy metals can also be complexed by organic compounds (ligands present in the effluents or/and cellular compounds); these soluble ligands can compete with the cells for the metals, reducing the efficiency of heavy metals removal (Simmons *et al.* 1995; Soares *et al.* 2002b; Soares *et al.* 2003). Redox potential can also affect the speciation of a given element; for example, chromium exists as Cr^{6+} or Cr^{3+} according to the E_h of the solution. The presence in solution of other cations besides the metals of interest can reduce heavy metals accumulation by biomass, by competition for binding sites on yeast cell wall (Norris and Kelly 1977; Ferraz and Teixeira 1999).

In recent years, the importance of chemical speciation in the treatment of wastewaters contaminated with heavy metals has been emphasized (Yun *et al.* 2001; Wang and Chen 2006; Gadd 2009). Romera *et al* (2008) compared the biosorption of different metal ions (Cd, Cu, Ni, Pb and Zn) by the marine brown alga *Fucus spiralis*; the predicted (theoretical) sorption values calculated using the speciation program PHREEQCI, were compared with experimental values. The influence of inorganic species distribution of Hg^{2+},Pt and Pd, as a function of the pH of the solution and of

chloride concentration, on the sorption capacity by the macroalga *Cystoseira baccata* (Herrero *et al.* 2005) and by sulfate-reducing bacteria (de Vargas *et al.* 2004) was also examined. However, all these works were carried out in simple water synthetic solutions. Real effluents are more complex matrices because they usually have multi-elements and inorganic ligands in competition; these aspects are particularly critical when nickel is one of the metal ions to be removed due to the low affinity of nickel to biomass (Pumpel *et al.* 2003; Machado *et al.* 2009).

In this work, the influence of the matrix of real effluents on the removal of heavy metals by bioremediation with heat-killed biomass of *Saccharomyces cerevisiae* was evaluated by analysing metals chemical speciation and validated for nickel through the bioremediation of a real electroplating effluent and two synthetic effluents. This work also explores the importance of metal chemical speciation in order to predict and optimize the treatment of real effluents, where multi-metal and multi-ligands can be present.

4.2. Materials and methods

4.2.1. *Strain, media and culture conditions*

In this work, the flocculent brewing strain of *Saccharomyces cerevisiae* National Collection of Yeast Culture (NCYC) 1364 was used.

Additional information about culture maintenance and pre-cultures, and cultures was described previously (Chapter 2, Section 2.2.1).

4.2.2. *Preparation of cell suspensions*

After growth, cells were harvested by centrifugation (2000 *g*, 5 minutes) and washed two times with 30 mM EDTA solution (Merck) and two times with deionised water. Cells were heat-inactivated by drying live biomass, at 45 ºC, until constant weight. In the previous work, it was found that all the biomass was dead after this treatment (Chapter 3). After being heat-killed, cells were washed five times with an excess of deionised water.

4.2.3. *Determination of biomass*

Cells concentration determination reported in this work was performed as described previously (Chapter 3, Section 3.2.3).

4.2.4. *Industrial effluent characterization*

In this study, an effluent from an electroplating industry from the Metropolitan Area of Oporto, Portugal, was used. To characterize this effluent, pH value, redox potential, total heavy metals (Cu, Ni, Zn and Cr), total inorganic ligands (chlorides, fluorides, phosphates, nitrates and sulphates) and total organic and inorganic carbon concentrations were determined. Inorganic ligands were analysed according to Standard Methods (APHA *et al.* 1998). All determinations were done at least in duplicate. Thus, fluorides were determined by direct potentiometry using a fluoride-ion-selective electrode (ISE) (Orion 96-09, Boston, USA), assembled to a Kent 7045 pH meter. Chlorides were determined by the argentometric method.

Nitrates were determined by direct potentiometry using a nitrate-ion-selective electrode (ISE) (Crison, Barcelona, Spain) assembled to a Crison MicropH 2002 pH

meter. The presence of interfering chemical species (such as: chlorides, nitrites, bicarbonate and complex organic acids), fluctuation of pH and ionic strength, were minimized by adding a buffer solution containing silver sulphate, sulfamic and boric acid, at pH 3.0.

Sulphates were determined by a turbidimetric method. To minimize the interference of suspended matter, effluents were filtered through a 0.45 μm pore size membrane. Colour and turbidity due to the matter < 0.45 μm were also corrected by preparing effluent blanks without $BaCl_2$.

Concentration of inorganic phosphate was determined by the molybdate method, using KH_2PO_4 (Merck) as a standard. The effluents were previously filtered through a 0.45 μm pore size membrane. For these determinations, glass material was firstly washed with hot diluted HCl.

Total carbon (TC) and inorganic carbon (IC) was determined using the analyser TOC-5000A (Shimadzu). Total organic carbon (TOC) was calculated by the difference of TC and IC.

Total heavy metals concentration was determined by AAS-FA in a Perkin Elmer AAnalyst 400 spectrometer (Norwalk, CT, USA), after appropriate dilution of the samples. Standard solutions for AAS calibration curves were prepared from a standard solution of 1000 mg/l (Merck) in 0.5 mM HNO_3 by dilution in deionized water. For this purpose, an intermediate solution of 100 mg/l was firstly prepared; then, five subsequent standard solutions were done. Each standard solution was read in triplicate. Cr^{6+} was determined by a colorimetric method (with diphenylcarbazide). The difference between total and hexavalent chromium was taken as trivalent chromium concentration.

4.2.5. *Preparation of the synthetic effluents*

Two synthetic effluents (named, synthetic 1 and synthetic 2), with the same amount of heavy metals present in industrial effluent, prepared from metal salt stock solutions of $Cu(NO_3)_2$, $NiCl_2$, $ZnCl_2$ and $Cr(NO_3)_3$, were produced in MES pH buffer (10 mM), at pH 6.0. In the case of synthetic effluent 1, the same amount of inorganic ligands (Cl^-, NO_3^- and SO_4^{2-}) of the industrial effluent was also added. MES is a suitable pH buffer for heavy metal studies because it does not complex with several metal ions, such as cadmium, copper, lead, and zinc (Soares *et al.* 1999a; Soares *et al.* 1999b).

4.2.6. *Computer chemical simulations*

All chemical speciation calculations were carried out using the MINEQL+ software (version 4.5) (Schecher and McAvoy 2003). Metal speciation analysis with MINEQL+ generates chemical equilibrium concentrations of all species being considered in the model by the program reactions, based on component stability constants and molar concentrations. For each case, total heavy metals and ligands (inorganic ligands quantified in the real effluent and inactivated biomass) concentrations, as well as all the affinity constants between heavy metals and ligands (Ferraz and Teixeira 1999; Martell and Smith 2004; Machado *et al.* 2009) and solubility product constants (Martell and Smith 2004) were introduced in the MINEQL+. The complexation parameters between heavy metals (Cu^{2+} or Ni^{2+} or Zn^{2+}) and biomass were determined in this Thesis (Chapter 3).

4.2.7. *Bioremediation of effluents using inactivated biomass in a batch reaction system*

The efficiency of using inactivated yeast biomass in the removal of heavy metals from electroplating effluents was tested using three effluents: one real and two synthetics.

Industrial effluent was adjusted to pH 6.0 with NaOH. Then, the effluent was allowed to stand undisturbed for one night and was filtered through a 0.45 μm pore size membrane.

Washed cells, with a concentration near 12 g dry weight/l, were added to the effluents and shaken in 500 ml plastic flasks, at 150 rpm, at 25 ºC. After 30 minutes of contact between the biomass and the effluent, cells were removed and the supernatant added to new yeast biomass, in a subsequent batch. Kinetic studies performed previously (Chapter 3) have shown that a contact time of 30 minutes was enough to reach the equilibrium between dead cells and metals in solution.

Control experiments were done incubating the effluents in the absence of biomass, in the same conditions described above; these control experiments showed that no appreciable amount of nickel (less than 1%) was adsorbed to the plastic flasks. Heavy metals remained in solution were determined as described above.

4.2.8. *Reproducibility of the results*

All experiments were performed in duplicate and repeated, at least, three times (n = 6).

4.3. Results and discussion

4.3.1. *Physico-chemical characterization of industrial effluent*

The real effluent was collected from a Portuguese electroplating industry, which is devoted to the production of bath accessories. Firstly, physicochemical characterization of the effluent was performed. For this purpose, total concentrations of metals, inorganic ligands, total carbon and total organic carbon were determined (Table 4.1); the permissible discharge levels for various heavy metals and inorganic ligands regulated by the Portuguese law (Decreto-lei 236/98) were included. European Union (EU) has some directives concerning the discharge of dangerous chemicals, which should be followed by the countries. However, all these documents are general guidelines without any limit discharge criteria. Thus, each country of the EU has its own laws, which take into account the communitarian directives. For comparative purposes and in order to give a wider regulatory perspective, the U.S.-Environmental Protection Agency (US-EPA 1984) criteria were also included.

The analysis of Table 4.1 evidences that nickel concentrations is above US-EPA and Portuguese limit discharge of effluents and thus should be removed before the effluent will be discharged. The presence of metals in the effluent is mainly due to periodical discharge of rinsing waters of electrolytic lines. The values of redox potential (238 mV) and pH (2.0) indicate that chromium should be present as Cr^{3+} (Pourbaix 1974), which is in the agreement with the obtained results (Table 4.1). Thus, the concentration of all other metals besides nickel (chromium, copper and zinc), as well as inorganic ligands, are below the legal limit of discharge.

Table 4.1. Physico-chemical characterization of a raw industrial effluent from an electroplating unit. The level of contaminants are compared to the Environmental Protection Agency (US-EPA 1984) and the Portuguese (Decreto-lei 236/98) wastewater limit discharge criteria.

	Wastewater limit discharge criteria		**Industrial effluent**
	US-EPA	**Portuguese law**	
pH (units)		6.0-9.0	2.0
E_h (mV)			238
TC (mg/l)			11.5
TOC (mg/l)			10.7
IC (mg/l)			0.8
Cl^- (mg/l)			90.5
F^- (mg/l)			12
NO_3^- (mg/l)		50	7.8
SO_4^{2-} (mg/l)		2000	374
PO_4^{3-} (mg/l)			0.08
Cu^{2+}			
(mg/l)	3.38	1.0	0.5
(μM)	53.1	16	8
Ni^{2+}			
(mg/l)	3.98	2.0	17.8
(μM)	67.8	34	303
Zn^{2+}			
(mg/l)	2.61	NS	0.8
(μM)	39.9	NS	12
Total Cr			
(mg/l)	2.77	2.0	1.2
(μM)	53.3	38	23
Cr^{6+}			
(mg/l)		0.1	<0.06
(μM)		2	<1

TC: total carbon. TOC: total organic carbon. IC: inorganic carbon.

Additionally, Table 4.1 also point outs that molar concentrations of chlorides and sulphates (2.55 mM and 3.89 mM, respectively), even if below the legal limits, are about ten times higher than those of nickel concentration (0.303 mM). The presence of these ligands in the electroplating effluents comes from the pickling and rinsing waters and, in some industries, from the addition, in the treatment tank, of an acid (chloridric acid) solution of bissulphite, which is used for reduction of Cr^{6+} to Cr^{3+} (Wang and Li 2006). Table 4.1 also shows that the amount of organic matter (quantified by the total organic carbon) and phosphates is negligible, whereas the concentrations of nitrates, fluoride and carbonates (expressed as IC) are of the same order of magnitude of nickel concentration.

4.3.2. O*ptimization of the bioremediation process*

The efficiency of bioremediation process of metal ions, from effluents, is dependent both on the chemical speciation of the metal ions present in the effluent and the accumulation parameters of biomass used: Q_{max} and log K; Q_{max} represents the maximum possible amount of metal ion adsorbed per unit weight of biomass and log K represents the equilibrium constant related to the affinity of the binding sites for the metals at a defined pH value. The most important factors which define metals speciation in the effluent are: pH, redox potential, concentration of metal ions and complexing agents (organic and inorganic ones), as well as their affinity for metal ions. This last parameter is of paramount importance, as it will be discussed below.

The values of Q_{max}, described in the literature, vary widely among the different types of biomass (bacteria, filamentous fungi, yeasts or algae) in evaluation and also within the same group of organisms (Wang and Chen 2009). This variability can be attributed to the different characteristics of the biomass, but also due to the different experimental conditions used, namely biomass concentration, pH of the solution and contact time between biomass and metals. As discussed in Chapter 3, the heat-

treated cells of *S. cerevisiae* used in the present work present intermediary values of metals accumulation (Cu, Ni and Zn), when compared with the values shown in others works, which have used *S. cerevisiae* cells (Machado *et al.* 2009).

For bioremediation treatment, pH 6.0 was chosen as being a good commitment solution between the efficiency of biomass to biosorb metal ions and the amount of hydroxide necessary to raise the pH of the effluent. Extremes of pH often decrease the accumulation of heavy metals by the biomass. Yeast cells of *S. cerevisiae* are able to remove heavy metals from wastewaters between pH 5.0 and 9.0 (Brady and Duncan 1994), being pH close to 5 - 6 as the optimal for Cu^{2+}, Cd^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+} and Cr^{3+} biosorption by yeast cells (Junghans and Straube 1991; Brady and Duncan 1994; Ferraz and Teixeira 1999; Özer and Özer 2003; Mapolelo and Torto 2004). At pH 6.0, the main chemical groups of biomass surface, that are able to participate in the removal of metal cations (carboxyl, phosphate, sulfydryl, hydroxyl and nitrogen-containing groups), are already totally (carboxylic acids) or partially (phosphate and amine groups) deprotonated (Martell and Smith 2004). As a consequence, good Q_{max} are obtained. On the other hand, pH 6 is the minimum pH value limit for discharging the wastewaters according to the Portuguese law (Table 4.1) and thus the pH of the effluents should be obligatorily raised up to 6 before being discharged. Additionally, if effluents also contain large amounts of other metal ions [e.g. Cu and Cr^{3+} ions], they will start to precipitate at this pH value, as metal hydroxides (Martell and Smith 2004), and thus more biomass will be available for removing nickel. However, the use of high pH values originates larger hydroxide consumptions and precipitation of other metals (e.g. Ni and Zn) hydroxides starts to occur (Martell and Smith 2004).

The major driving force for the success of an efficient bioremediation treatment of an effluent containing metal ions is the relative affinity of each metal for biomass versus the several ligands (inorganic and organic ligands) present in the effluent, which can compete with yeast cells. Thus, in order to optimize the

conditions of the bioremediation process, metal affinity constants among metal ions and inorganic ligands and yeast cells of *S. cerevisiae* were taking into account (Table 4.2).

For all metals, unless chromium, affinity constants of the aquocomplex metal ion, $M^{2+}_{(aq.)}$ were considered. In the case of chromium, this metal ion is very acidic and starts to hydrolysis at about pH 2; in an aqueous solution, $Cr(OH)^{2+}_{(aq)}$ is the major species present in solution before precipitation of $Cr(OH)_3$ occurs and this is the reason why we have included the affinity constants for this component in Table 4.2. A diagram exemplifying the competitive effect of ligands, present in the effluent, with yeast cells biomass is shown in Figure 4.1.

Table 4.2. Equilibrium affinity constants, as log K, among the different ligands and copper, nickel and zinc, at pH 6.0, and chromium at pH 5.0*.

	log K				
Ligand	**$Cr(OH)^{2+}$**	**Cu^{2+}**	**Ni^{2+}**	**Zn^{2+}**	**Reference**
Biomass	3.56				Ferraz *et al.* (1999)
		3.99	3.34	3.59	Machado *et al.* (2009)
Cl^-	3.77[a)]	0.20[b)]	0.41[b)]	0.40[b)]	
F^-	8.86[a)]	1.80[b)]	1.40[b)]	1.30[b)]	
NO_3^-	2.30[a)]	0.50[b)]	0.40[b)]	0.40[b)]	
HCO_3^-	-	-3.56c)	-	-	Herrero *et al.* (2005)
		1.80[b)]	2.10[b)]	1.50[b)]	
SO_4^{2-}	2.38[d)]	2.36[b)]	2.30[b)]	2.34[b)]	
$H_2PO_4^-$	6.42[a)]	-	-	-	

* Conditional affinity constants were calculated assuming the following equilibriums, wherein M is metal and L is ligand:

a) $Cr(OH)^{2+} + L + H^+ \leftrightarrows CrL + H_2O$

b) $M + L \leftrightarrows ML$

c) $Cu^{2+} + HCO_3^- \leftrightarrows CuCO_3 + H^+$

d) $Cr(OH)^{2+} + SO_4^{2-} \leftrightarrows CrOHSO_4$

A diagram exemplifying the competitive effect of ligands, present in the effluent, with yeast cells biomass is shown in Figure 4.1.

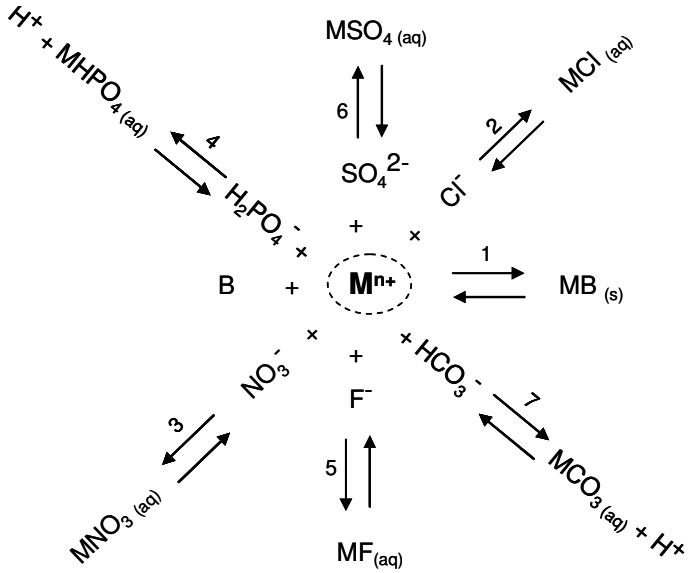

Figure 4.1. Diagrammatic representation of metal-ligands interactions. Metal ions: M^{n+}. Biomass (yeast cells): B. For simplicity of representation, the charges of the complexes were omitted. Yeast cells are able to remove heavy metals from industrial effluents (reaction 1). The presence of ligands in the effluents (reactions 2 - 6) can reduce the efficiency of the process by competing with biomass for metal removal.

The analysis of Table 4.2 shows that, for copper, nickel and zinc, the affinity constants between metal ions and yeast biomass are much higher (two decades or more) than with some inorganic ligands. The relative magnitude of the affinity constants indicate that carbonates, chlorides, fluorides and nitrates do not compete with biomass for these metal ions even if present in large excess. In practice, $MHCO_3$, MCl, MF and MNO_3 species, corresponding to reactions 2, 3, 4 and 5, respectively (Figure 4.1), are not formed in appreciable extension; thus, accumulation of copper and/or nickel and/or zinc ions by yeast cells (reaction 1) in effluents containing carbonates and/or chlorides and/or fluorides and/or nitrates, would not

be influenced by the presence of these ligands. Table 4.2 also shows that the affinity constants between copper or zinc ions and yeast biomass are much higher (two decades or more) than with sulphates.

On the other hand, the relative magnitude of the affinity constants between nickel ions and sulphates or biomass only differs one decade. These values anticipate that effluents containing molar concentrations of sulphates much higher than that of nickel ions can compete with yeast cells and thus will reduce the bioremediation efficiency (reaction 6). However, this effect can be more or less pronounced depending on the amount of biomass used. Theoretical simulations of the bioremediation process using 1.5 g/l of biomass, of an effluent with the same composition of the effluent described in Table 4.1, and the same effluent but with a concentration of sulphates ten times higher (3740 mg/l), predict a reduction of 24% of the bioremediation efficiency, i.e., the % of nickel associated with the biomass (Figure 4.2); for the lower concentration of sulphates (374 mg/l), the fraction of nickel associated with biomass is 35%, reducing to 11% in the presence of a concentration of sulphates ten times higher (Figure 4.2).

High concentrations of sulphates can occur in these type of effluents when reduction of Cr^{6+} to Cr^{3+} is performed with a sulphuric acid solution of bissulphite. Taking into account the negative impact of sulphates in the bioremediation of nickel (Figure 4.2), the use of sulphuric acid should be substituted by another mineral acid, for example nitric or chloridric acid, when acidification is necessary for previous reduction of Cr^{6+} to Cr^{3+}. Besides, addition of bissulphite in the treatment tank can be reduced by controlling the redox potential and the pH; usually a redox potential of 270 mV and a pH of 2.5 are enough to reduce efficiently Cr^{6+} to Cr^{3+} (Pourbaix 1974).

The analysis of the relative magnitude of the affinity constants among chromium(III) and biomass or the various inorganic ligands (Table 4.2), anticipate

that, effluents containing fluorides or phosphates complex chromium (reactions 4 and 7, respectively), and thus, the biosorption process under these conditions can be reduced (Figure 4.1).

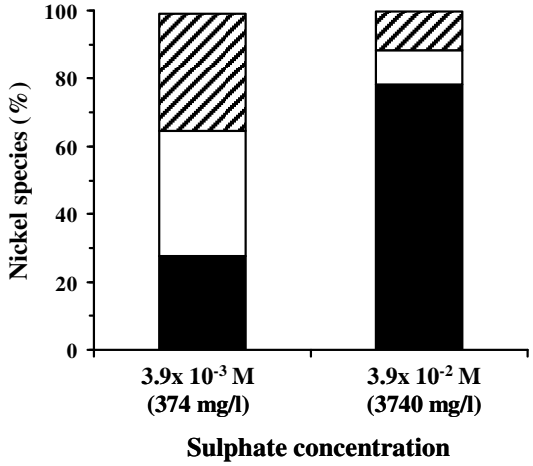

Figure 4.2. Simulation of the influence of sulphate concentration on the nickel speciation in the presence of 1.5 g/l of inactivated biomass of *S. cerevisiae* NCYC 1364. Free nickel ions (white part of the bar); nickel associated with biomass (dashed part of the bar); nickel associated with sulphate (black part of the bar). The different species of nickel were calculated using the chemical equilibrium computer program (MINEQL+); for more details about calculations see section computer chemical simulations.

Copper, chromium, nickel and zinc are the metal ions most widely found in the electroplating effluents and the affinity between biomass used in this work and these metal ions follows the following order: Cu >> Cr ≅ Zn > Ni (values of log K are presented in Table 4.2) (Ferraz and Teixeira 1999; Machado *et al.* 2009). The treatment of effluents containing multi-elements is more complex because metal ions compete for binding sites; as a consequence, displacement of one metal species by another, which has higher affinity for biomass binding sites, can occur (Brady *et al.* 1994). Thus, for an efficient bioremediation process, the amount of biomass ([Biomass]) when compared with the total amount of metals (∑ [M]) should be in excess in order to have sufficient available biomass sites for all metals accumulation, including those with lower affinity for biomass. However, as lower as the affinity

constants between biomass and metal ions are, less amount of metal ions will be biosorbed when equilibrium is attained. Since biomass presents much lower affinity to nickel than copper (five times less) and chromium or zinc (two times less) (Table 4.2), this means that the efficiency of bioremediation of effluents containing nickel, among the other three metal ions (chromium, copper and zinc), will be conditioned by nickel affinity.

If we want to remove efficiently metals from real multi-metal-systems, which contain nickel among other metal ions, nickel biosorption is one of the real challenges that should be firstly studied. Due to this, we decided to study in detail the biosorption of real electroplating effluent containing this metal.

In order to optimize the amount of yeast cells required to treat the effluent described in Table 4.1, in a batch mode, with no more than three batches, theoretical calculations were done using MINEQL+ software (Schecher and McAvoy 2003). Thus, for predicting the metal accumulated by the yeast cells, an iterative process of chemical simulations was performed; for this purpose, the program was run with a fixed concentration of biomass in the range between 1.5 and 12 g/l, at pH 6.0, assuming the chemical composition of the effluent (Table 4.1). The analysis of Figure 4.3-A predicts that the application of 1.5 g/l of biomass will remove about a third of nickel concentration in the first batch. After the fifth batch, the amount of nickel (free nickel ion plus $NiSO_4$) present in the effluent will be below the limit of discharge criteria according to the Portuguese law.

On the other hand, chemical simulations performed with 12 g/l of yeast cells, in each batch, predicted the removal of about 84% of nickel in the first batch; according to these calculations, after the second batch, the concentration of nickel (free nickel ion plus $NiSO_4$) in the effluent will be below the limit of discharge criteria (Figure 4.3-B). Amounts of 1.5 and 12 g/l of biomass correspond to molar concentrations of 5.4×10^{-4} and 4.5×10^{-3} M (Machado *et al.* 2009), respectively. For

these experimental conditions, the molar ratios between the concentration of biomass and nickel are 1.8 and 14.9, respectively. These results evidence that a large excess of biomass in relation to the total molar amount of metals present in the effluents should be used to overcome the lower affinity of biomass to nickel and sulphates competition.

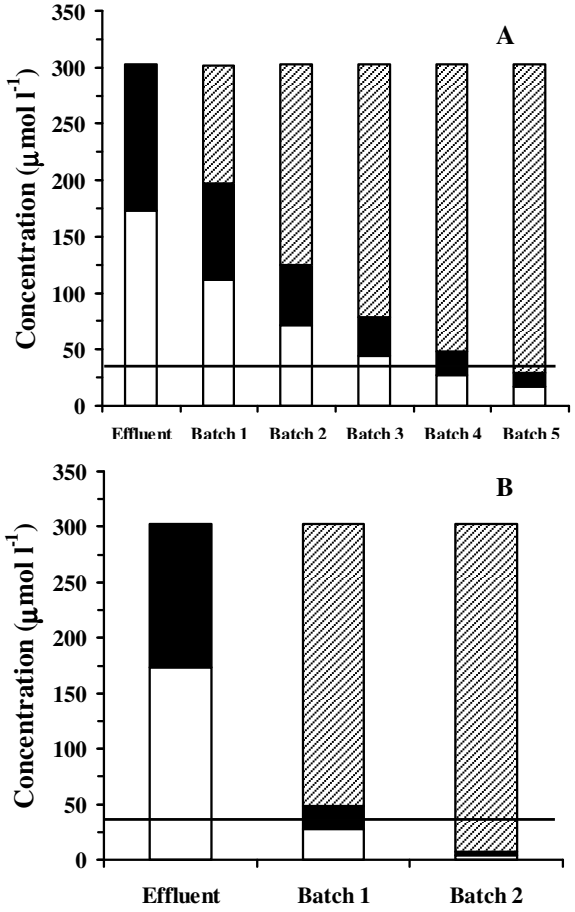

Figure 4.3. Simulation of the influence of yeast biomass concentration in the nickel removal. A - 1.5 g dry weight/l of yeast biomass; B – 12 g dry weight/l of yeast biomass. Nickel ions (white part of the bar); nickel associated with inactivated yeast of *S. cerevisiae* NCYC 1364 (dashed part of the bar); nickel associated with sulphate (black part of the bar). Calculations of the nickel concentration species was done as described in Figure 4.2. Solid line represents the Portuguese wastewater limit discharge criteria for nickel: 2.0 mg/l (34 µM).

4.3.3. *Bioremediation of real and synthetic effluents using a batch reaction system*

In this work, inactivated (at 45 ºC) yeast cells of a brewing strain of *Saccharomyces cerevisiae* were used in the treatment of a real electroplating effluent containing nickel above the legal limit of discharge criteria; in order to compare the impact of inorganic ligands, present in the raw effluent, on the bioremediation process, two synthetic effluents (named, synthetic effluent 1 and synthetic effluent 2) with the same amount of metals as the industrial effluent were prepared. In the case of synthetic effluent 1, the same amount of inorganic ligands (Cl^-, NO_3^- and SO_4^{2-}) of the industrial effluent was also added.

Taking into account the information given above, 12 g/l of yeast cells were used to remove nickel from the effluents, in a batch mode. A series of batches, using new biomass in each batch, was implemented in the effluents (real and synthetics). Final concentrations of nickel and cumulative percentages of nickel removal obtained, after each batch in all effluents, are present in Table 4.3 and Figure 4.4, respectively. Theoretical simulation of cumulative percentages of nickel removal were also calculated and included in Figure 4.4; for this purpose, chemical speciation of nickel at pH 6.0 was calculated, assuming the total concentration of nickel, biomass and inorganic ligands present in the effluent, as well as the affinity constants between nickel and the ligands (biomass and inorganic ligands) using the MINEQL+ software (Schecher and McAvoy 2003).

Table 4.3. Bioremediation of industrial and synthetic effluents using *S. cerevisiae* NCYC 1364, in a batch mode reaction. The level of the metal reached is compared to the Environmental Protection Agency (US-EPA 1984) and the Portuguese (Decreto-lei 236/98) wastewater limit discharge criteria.

		Metal concentration (mg/l)[#]					
	Effluent	**Limit discharge criteria**		**Original effluent**	**After 1st batch**	**After 2nd batch**	**After 3rd batch**
		US-EPA	**Portuguese law**				
Nickel	**Industrial**	3.98	2.0	17.8 ± 0.1	5.4 ± 0.5	2.7 ± 0.7	2 ± 1
	Synthetic 1			19 ± 1	6.7 ± 0.3	3.2 ± 0.4	2.2 ± 0.3
	Synthetic 2			19± 1	5.3 ± 0.4	2.6 ± 0.4	1.6 ± 0.3

[#] Mean and standard deviation of three independent experiments performed in duplicate *(n=6)*.
Synthetic effluent 1 and 2 had the same amount of heavy metals present in industrial effluent and were prepared in MES pH buffer, at pH 6.0. In the case of synthetic effluent 1, the same amount of inorganic ligands (Cl^-, NO_3^- and SO_4^{2-}) of the industrial effluent was also added.

After the first batch, the percentages of nickel removal were 70, 65 and 72% (Figure 4.4), for real and synthetic effluents with and without ligands, respectively. In the second batch, the replacement of biomass lowered the amount of nickel in solution to 2.7, 3.2 and 2.6 mg/l in real and synthetic effluents, respectively (Table 3.3). After the third batch, the concentration of nickel lowered to 2, 2.2 and 1.6 mg/l in real and synthetic effluents (Table 3.3), which corresponded to 89, 88 and 92% of total cumulative removals, respectively (Figure 4.4). This means that, after the implementation of the bioremediation process described in the present work, the concentration of nickel in the effluents reached the quality criteria for industrial effluents discharge (3.98 and 2.0 mg/l), after the second or third batch according to U.S.-Environmental Protection Agency (US-EPA 1984) and Portuguese law (Decreto-lei 236/98) respectively.

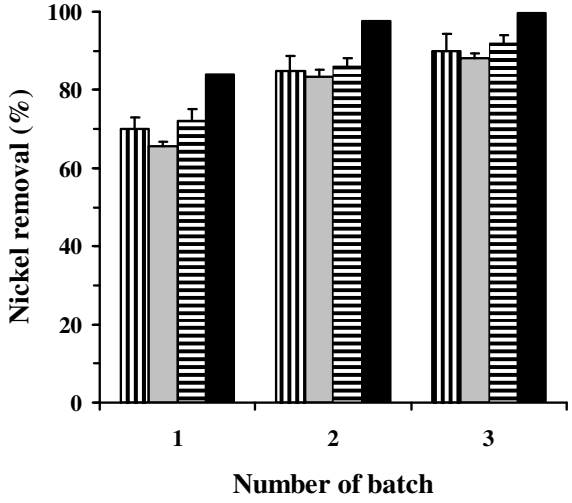

Figure 4.4. Comparison of the theoretical and real nickel removal by *S. cerevisiae* NCYC 1364 through a series of sequential batches in bench scale experiments. Bar with vertical lines: industrial effluent; grey bar: synthetic effluent 1 (with the same amount of inorganic ligands of industrial effluent); bar with horizontal lines: synthetic effluent 2 (without addition of inorganic ligands); black bar: theoretical value. Theoretical calculations were performed as described in Figure 4.2. Experimental assays were carried out with 12 g dry weight/l of biomass added to the effluents reported in Table 4.3. The effluents plus the biomass were placed in plastic flasks and shaken at 150 rpm, at 25 ºC. After 30 minutes of contact of the biomass with the effluents, cells were removed and the supernatant added to a fresh yeast biomass, in a subsequent batch. Values are the mean and standard deviation of two replicates of three independent experiments (n=6).

The comparative analysis of these results shows that equivalent nickel removal values were obtained from all effluents. Together, these results are in agreement with our prevision that the amount of inorganic ligands, as well as the small amount of organic matter, present in the real effluent did not influence the nickel removal.

Experimental values of nickel removal from effluents were lower than the theoretical simulated values (Figure 4.4). The overestimation of the amount of nickel accumulated by the biomass can be a consequence of the stability constants between nickel and inorganic ligands used in the theoretical simulation calculations were not determined in the experimental conditions of the effluents (ionic strength and temperature) and/or due to the release of some ligands by the biomass, which will complex nickel and thus reduce the efficiency of bioremediation process (Soares *et al.* 2002b; Soares *et al.* 2003).

These results evidence that the use of a large excess of yeast biomass comparatively to nickel allowed an efficient removal of nickel from real and synthetic effluents, reducing the amount of metal in solution to the limit of discharge of wastewater in natural waters.

4.4. Conclusions

This work examines the influence of the competitive effect of inorganic ligands (carbonates, chlorides, fluorides, phosphates, nitrates and sulphates), which can be present in real electroplating effluents, on the biosorption of chromium, copper, nickel and zinc ions by yeast cells of *S. cerevisiae*. The presence of high concentrations of carbonates, chlorides, fluorides, and nitrates do not compete with biomass for copper, nickel and zinc ions. On the other hand, the presence of phosphates or fluorides can reduce the efficiency of chromium biosorption. The presence of sulphates, at molar concentrations higher than that of biomass, can compete with

biomass for nickel. To avoid high concentrations of sulphates in the electroplating effluents, and thus the problem of sulphates competition, we suggest the substitution of sulphuric by nitric acid (or chloridric acid) in the bissulphite solution for reduction of Cr^{6+} to Cr^{3+} in the wastewater treatment plants of the electroplating industries.

Theoretical simulations, validated experimentally, have shown that for the bioremediation treatment of effluents containing nickel at pH 6.0, using a serial batch reactor, the amount of biomass ([Biomass]), when compared with the total amount of metals ($\sum$ [M]) should be in excess. We have demonstrated that the molar ratio of [Biomass]/($\sum$ [M]) to be implemented can be computationally predicted, with an acceptable precision, if the concentrations of nickel and sulphates present in the effluent, as well as the affinity parameters (Q_{max} and log K) of the biosorbent, are known.

Finally, brewing flocculent cells of *S. cerevisiae* seems to be a promissory biomass alternative for the removal of nickel from industrial electroplating effluents. After the third batch, the amount of nickel in the effluent was reduced to values below the limit of discharge of wastewater in natural waters. This corresponded to a removal of nickel of 89%.

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Chapter 5

Impact of fluorides on the removal of heavy metals from an electroplating effluent using a flocculent brewer's yeast strain of *Saccharomyces cerevisiae*[4]

Abstract

Besides several toxic heavy metals, real electroplating effluents can have in solution different cations and anions, which may influence heavy metals removal by the biomass. In the present work, the effect of the presence of fluorides in the efficiency of Cr^{3+}, Cu^{2+} and Ni^{2+} removal, from a real effluent, by heat-inactivated cells of a brewing flocculent strain of *Saccharomyces cerevisiae* was evaluated. Fluorides are commonly used in the electroplating industries and thus can be found in the respective wastewaters. The presence of fluorides severely decreased the removal of Cr^{3+} by yeast biomass; conversely, a higher removal of Cu^{2+} and Ni^{2+} was observed. This behaviour can be understood by metals speciation. In the presence of fluorides, Cr^{3+} was mainly complexed, becoming unavailable for yeast accumulation, which decreased the efficiency of Cr^{3+} removal. Thus, in the presence of fluorides less Cr^{3+} is associated with biomass and consequently more yeast binding sites remain available for the uptake of other metals present in solution. This fact can explain the increase of Cu^{2+} and Ni^{2+} removal in the presence of fluorides.

5.1. Introduction

Electroplating industries produce large amounts of effluents containing several heavy metals. These wastewaters must be treated before being discharged into the aquatic systems to prevent serious environmental dangerous.

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[4] Submitted for publication

The accumulation of heavy metals by dead biomass (biosorption) is attributed to the interactions of yeast cell surface with the metals, in a metabolism-independent way, involving, most likely, one or more of the following mechanisms: adsorption, complexation, precipitation and crystallization with the microfibrillar yeast cell wall structure (Gadd 1990). Metals removal by inactivated biomass is influenced by the physico-chemical characteristics of the solution, namely pH, E_h and the presence of organic or inorganic ligands (Gadd 1993; Naja *et al.* 2010). These factors also determine metal speciation (the different physicochemical forms of the metal in solution) and therefore the available metal (i.e., the free and labile metal) (Florence 1983).

Effluents from the electroplating industries contain, besides the toxic heavy metals, other pollutants, such as organic (ethylenediaminetetraacetic acid, EDTA) or inorganic ligands (carbonates, phosphates, fluorides and cyanides) (Naja and Volesky 2010). In a general way, the presence of ligands reduce metal accumulation by the biomass, being this effect dependent of the chemical speciation of the metal ions present in the effluent and the accumulation parameters of biomass used. Thus, an inhibition of gold accumulation by the unicellular algae *Chlorella vulgaris* in the presence of chloride, bromide and iodide was reported (Greene *et al.* 1986). Conversely, Diniz and Volesky (2005) described that chloride and nitrate seems not to affect La^{3+} sorption by the brown seaweed *Sargassum polycystum,* while sulphate decrease the metal removal. On the other hand, Ahuja et al. (1999) reported a drastic reduction of Co^{2+} uptake by the freshwater cyanobacterium *Oscillatoria anguistissima* due to the presence of sulphate and nitrate. The inhibition of metal uptake is more pronounced when ligands with strong complexing power, like mercaptoethanol, cyanide or thiourea (Greene *et al.* 1986), are present in solution. Tobin et al. (1987) described a total inhibition of Cd^{2+} and Pb^{2+} uptake by the fungus *Rhizopus arrhizus* due to the presence of EDTA. Similarly, Ramelow et al. (1992) have shown inhibition of heavy metals uptake by seaweed biomass in the presence of EDTA;

other organic ligands had a lesser (sodium citrate) or no (sodium lauryl sulphate) significant effect.

Among the different ligands that can be found in electroplating effluents, fluorides are very common as they are used for pickling certain metal alloys and in some chromium electrodeposition baths (Schlesinger 2004).

The present work aims to evaluate the effect of the presence of fluorides in the efficiency of heavy metals [Cr^{3+}, Cu^{2+} and Ni^{2+}] removal from a real electroplating effluent, using heat-inactivated cells of the brewing flocculent strain of *Saccharomyces cerevisiae*.

5.2. Materials and methods

5.2.1. *Strains, media and culture conditions*

The flocculent brewing strain of *Saccharomyces cerevisiae* National Collection of Yeast Culture (NCYC) 1364 was used in this work.

Additional information about culture maintenance and pre-cultures, and cultures was described previously (Chapter 2, Section 2.2.1).

5.2.2. *Preparation of cells suspension*

After growth, cells were harvested by centrifugation (2000 *g*, 5 minutes), washed two times with 30 mM ethylenediaminetetraacetic acid (EDTA) solution and two times with deionised water. Cells were heat-inactivated by drying live biomass at 45 ºC. After being heat-killed, cells were washed five times with an excess of

deionised water. Cell concentration was determined as described previously (Chapter 3, Section 3.2.3).

5.2.3. *Metals quantification*

Total heavy metals and Cr^{6+} concentrations were determined as described in Chapter 4, Section 4.2.4.

5.2.4. *Chemical speciation studies*

Chemical speciation calculations were carried out as described in Chapter 4, Section 4.2.6. Computational simulations were performed at pH 6.0, considering the total heavy metals, fluorides and biomass concentrations, as well as all the affinity constants between heavy metals and fluorides or biomass (Ferraz and Teixeira 1999; Martell and Smith 2004; Machado *et al.* 2009) and solubility product constants (Martell and Smith 2004). The complexation parameters between heavy metals (Cu^{2+} or Ni^{2+} or Zn^{2+}) and biomass were determined in this Thesis (Chapter 3).

5.2.5. *Bioremediation of the effluent*

The industrial effluent was doped with sodium fluoride in a final concentration of 2.78 g/l. Then, the effluent was treated with an excess of sodium bisulphite to guarantee the reduction of Cr^{6+} to Cr^{3+}. The efficiency of this process was controlled by the determination of Cr^{6+}, as described in Section 4.2.4. Subsequently, the effluent was diluted four times and the pH was adjusted to 6.0 with NaOH; the effluent was allowed to stand undisturbed for one night and then filtrated through a 0.45 μm-pore size membrane.

Washed cells, with a concentration near 18 g dry weight/l, were added to the effluent and shaken in 500 ml plastic flasks at 150 rpm and 25 ºC. After 30 minutes of contact between the biomass and the effluent, cells were removed and the supernatant added to new yeast biomass, in a subsequent batch. Kinetics studies previously described (Chapter 3) have shown that a contact time of 30 minutes was enough to reach the equilibrium between dead cells and metals solution.

Control experiments were done incubating the effluent in the absence of biomass, in the same conditions described above; these control experiments showed that no appreciable amount of metals (less than 2%, for all metals in the effluent) was adsorbed to the plastic flasks. Heavy metals concentrations were determined as described in Chapter 4, Section 4.2.4.

5.2.6. *Reproducibility of the results*

All experiments were performed in duplicate and repeated independently at least twice ($n = 4$).

5.3. Results and discussion

A large body of heavy metals bioremediation work, using *Saccharomyces cerevisiae* cells, has been carried out in synthetic solutions, which simplify the systems under study. The application of yeast cells, or other types of biomass, to treat real effluents, is very scarce (Gadd 2009). The simultaneous presence of several heavy metals in solution, associated to the presence of organic and inorganic ligands, difficult much more the treatment of real effluents.

In the present work, a real effluent originated from the pickling and chromium-nickel lines from a Portuguese electroplating industry was used (effluent B used in Chapter 6). The effluent presented Cr^{3+} and Ni^{2+} concentrations above the limits of discharge allowed by the US-Environmental Protection Agency (US-EPA 1984) and the Portuguese Law (Decreto-lei 236/98) (Table 5.1); according to the Portuguese law, Cu^{2+} was also in excess (Table 5.1). For further details about its chemical composition see effluent B in Table 6.1, Chapter 6.

Fluorides are very commonly used in the electroplating industries (Schlesinger 2004) and, therefore, can be found in the respective wastewaters. In Table 5.2, the affinity constants between the metal ions present in the real effluent (Cr^{3+}, Cu^{2+} and Ni^{2+}) and the yeast cells used in the present work, as well as the affinity constants among heavy metals and fluorides, are present. The magnitude of the affinity constants between Ni^{2+} and Cu^{2+} and fluorides is about two decades lower than those observed between metals and biomass (Table 5.2). These data anticipate that fluorides do not compete with biomass for these metal ions even if present in large excess. On the other hand, Table 5.2 shows that the magnitude of the affinity constants between Cr^{3+} and fluorides is about five decades higher than those observed between Cr^{3+} and biomass. Therefore, effluents containing molar concentration of fluorides similar to those of Cr^{3+} will compete with yeast cells and reduce or inhibit the bioremediation efficiency.

In order to evaluate the effect of the presence of fluorides in the bioremediation process, the original effluent was doped with fluorides. As it can be seen it Table 5.1, the presence of fluorides increases Cr^{3+} concentration, in solution, at pH 6.0; most likely, the complexation of Cr^{3+} by fluorides avoids its precipitation at pH 6.0. In addition, the presence of fluorides impairs deeply the removal of Cr^{3+} by yeast cells (Table 5.1).

Table 5.1. Effect of fluoride ions on the bioremediation of an industrial effluent using *Saccharomyces cerevisiae* NCYC 1364 in a batch mode reaction.

	Presence of fluoride	Metal concentration (mg/l)[a]					
		Limit discharge criteria		Effluent[b]	After 1st batch	After 2nd batch	After 3rd batch
		US-EPA	Portuguese law				
Total Cr	with	2.77	2.0	24 ± 3	21 ± 1	18.2 ± 0.8	17 ± 1
	without			15 ± 3	5 ± 1	1.9 ± 0.2	1.2 ± 0.2
Cu(II)	with	3.38	1.0	1.7 ± 0.3	0.2 ± 0.1	ND	ND
	without			1.5 ± 0.4	0.1 ± 0.1	ND	ND
Ni(II)	with	3.98	2.0	21.1 ± 0.4	6.8 ± 0.4	2.0 ± 0.1	0.8 ± 0.1
	without			21 ± 1	8.6 ± 0.7	3.5 ± 0.5	2.2 ± 0.4
Zn(II)	with	2.61	NS	1.2 ± 0.1	ND	ND	ND
	without			1.0 ± 0.1	ND	ND	ND

The level of the metals reached are compared with the same effluent without fluorides (Chapter 6, Effluent B), the US-Environmental Protection Agency (US-EPA 1984) and the Portuguese (Decreto-lei 236/98) wastewater limit discharge criteria.
ND: not determined; metal concentration was below the legal limit of discharge. NS: not specified in the law.
[a]Mean and standard deviation of two independent experiments performed in duplicate *(n=4)*.
[b]After chromium(VI) reduction with bisulphide, dilution (4 times) and pH adjustment to 6.0.

Table 5.2. Equilibrium affinity constants.

Ligand	log K			Reference
	$Cr(OH)_2^+$	Cu^{2+}	Ni^{2+}	
Yeast cells	3.56			Ferraz and Teixeira (1999)
		3.99	3.34	Machado *et al.* (2009)
F^-	8.86[a]	1.80[b]	1.40[b]	Schecher and Mcavoy (2003)

Conditional affinity constants, log K, among the different ligands (yeast cells of *Saccharomyces cerevisiae* and fluorides) and copper or nickel, at pH 6.0, or chromium at pH 5.0. Constants were calculated assuming the following equilibriums, wherein M is metal and L is ligand:

[a] $Cr(OH)^{2+} + L + H^+ \leftrightarrows CrL + H_2O$

[b] $M + L \leftrightarrows ML$

For comparative purposes, the treatment of the same industrial effluent, without fluorides, is shown. In the absence of fluorides (Table 5.1), the industrial effluent was treated after the third batch, i.e., it meets the quality discharge criteria for all the metals [Cr^{3+}, Cu^{2+} and Ni^{2+}], according to US-EPA (US-EPA 1984) and the Portuguese law (Decreto-lei 236/98). Conversely, in the presence of fluorides, an effective treatment of the effluent was not observed; after the third batch, a removal of Cr^{3+} lesser than 30% was achieved (Figure 5.1). Interestingly, in the presence of fluorides, the efficiency of Ni^{2+} removal was enhanced (Table 5.1). After the second batch, 91% of Ni^{2+} was removed, while, in the absence of fluorides, similar values were only observed after the third batch (Figure 5.1).

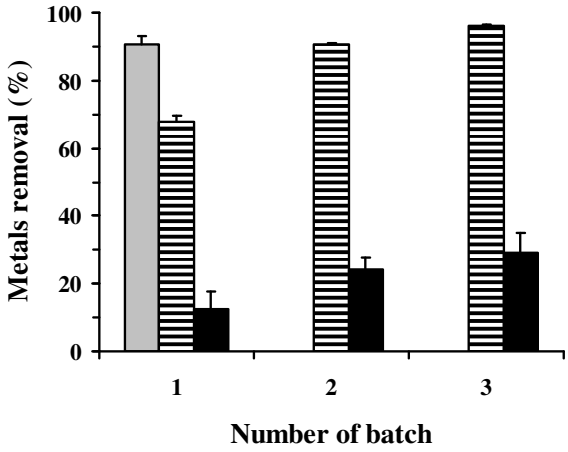

Figure 5.1. Evolution of the % of metals removal by heat-inactivated cells of *Saccharomyces cerevisiae* NCYC 1364 through a series of sequential batches. Metals: Cu^{2+} (grey bar); Ni^{2+} (bar with horizontal lines); Cr^{3+} (dark bar). Batch assays were carried out with 18 g/l of dry weight biomass, at pH 6.0. The effluent plus the biomass were placed in plastic flasks and shaken at 150 rpm, at 25 ºC. After 30 minutes of contact between the biomass and the effluent, cells were removed and the supernatant added to a fresh yeast biomass, in a subsequent batch. Values are the mean of two replicates of two independent experiments; standard deviations are presented (n = 4).

The effect of fluorides on metals removal by yeast biomass can be explained taking into account the distribution of the metals species in the presence or absence of fluorides (Figure 5.2). Fluorides have much higher Cr^{3+} affinity than yeast cells (Table 5.2); thus, when present in solution, it complexes the majority of Cr^{3+} (66%, Figure 5.2-A). The competitive effect of fluorides leads to a decrease of Cr^{3+} associated with biomass (31%) (Figure 5.2-A), when compared in its absence (91%) (Figure 5.2-B). As a consequence, a reduction of the bioremediation efficiency occurs (Table 5.1 and Figure 5.1).

On the other hand, biomass has higher affinity for Cu^{2+} and Ni^{2+} than fluorides (Table 5.2). Therefore, fluorides do not compete (or its effect is reduced) with biomass for Cu^{2+} and Ni^{2+} available in solution. The distribution diagrams for these metals show that lesser than 10 % of Cu^{2+} and Ni^{2+} are complexed with

fluorides (Figure 5.2-A), while the percentages of metals associated with the biomass are similar in those in the absence of fluorides (Figure 5.2-B).

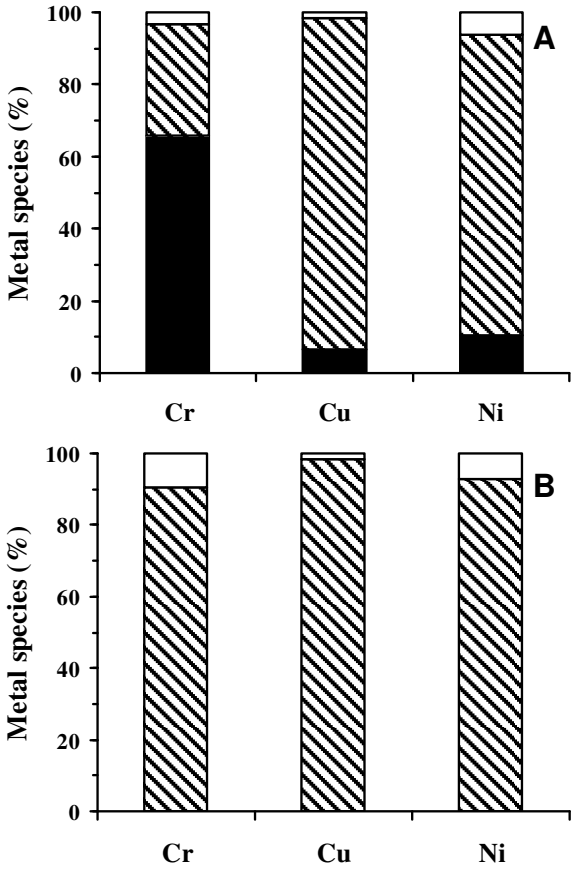

Figure 5.2. Effect of fluoride in the speciation of Cr^{3+}, Cu^{2+} and Ni^{2+}. Industrial effluent with (**A**) or without (**B**) fluoride ions. Free metals ions (white part of the bar); metals associated with biomass (dashed part of the bar); metals associated with fluoride (black part of the bar). The different species in solution were calculated using a chemical equilibrium computer program (MINEQL+); for more details about calculations see Section 5.2.4.

Chemical speciation studies are in agreement with the experimental results. When fluorides are present, Cr^{3+} is mainly complexed by fluorides, which reduces the available chromium to be removed by the yeast cells. However, for the same amount of biomass, in the absence of available Cr^{3+}, more yeast binding sites are vacant for

removing other metal (Cu^{2+} and Ni^{2+}) ions. These facts explain the increase of Ni^{2+} removal efficiency in the presence of fluorides. This aspect is particularly remarkable for metals, which biomass displays a lower affinity, as it is the case of Ni^{2+}.

5.4. Conclusions

In the present work, it was shown that the presence of fluoride anions in electroplating effluents may decrease the efficiency, or even preclude, of bioremediation of Cr^{3+} by yeast cells. This effect can be attributed to the complexation of Cr^{3+} by fluorides. However, the presence of fluorides does not affect the bioremediation of effluents containing Cu^{2+} and Ni^{2+} by *Saccharomyces cerevisiae* heat-inactivated yeast cells.

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Chapter 6

Removal of heavy metals using a brewer's yeast strain of *Saccharomyces cerevisiae*: application to the treatment of real electroplating effluents containing multielements[5]

Abstract

Yeast cells have been recognized as an effective type of biomass for the treatment of wastewaters containing heavy metals. However, its capability to treat efficiently complex effluents loaded with several metals ions (Cr, Cu, Ni and Zn) has never been reported. The aim of this work was to study the feasibility of a hybrid technology, which combines chemical precipitation at pH 6.0 with a subsequent biotechnological-based process (using heat-killed cells of a flocculent brewing strain of *Saccharomyces cerevisiae*), to remove simultaneously several metals from real electroplating effluents. Two effluents containing Cu, Ni and Zn (effluent A) or Cr, Cu and Ni (effluent B) were treated. In both effluents, pH was adjusted to 6.0; in effluent B, Cr^{6+} was previously reduced to Cr^{3+}. Chemical speciation studies allowed defining the amount of biomass to be employed with a minimum number of batches. Subsequently to pH adjustment to 6.0, effluents were fully treated with a serial batch of biomass. After the third batch, metal concentrations were lowered to below the legal limits of discharge; removals $\geq$ 89, 91, 92 and 94% were attained for Ni, Cu, Cr and Zn, respectively. In the present work, the usefulness of using flocculent brewing yeast cells to treat complex industrial effluents loaded with several heavy metals was demonstrated. The hybrid process developed showed to be an efficient alternative for the treatment of real electroplating effluents.

[5] Journal of Chemical Technology and Biotechnology DOI 10.1002/jctb.2440

6.1. Introduction

Metal pollution due to the release of wastewaters from electroplating, metallurgical or tanning industries, is one of the major concerns in industrialized countries. Electroplating is an industrial growth sector (Volesky 2003). The process consists in a deposition of a thin layer of metal using electrical current, upon a desired material, for improving the surface properties. The articles to be treated are immersed in aqueous solution containing the metal to be plated as well as other ions (e.g. carbonates, cyanides and phosphates) to increase conductivity. After plating, the articles are washed in a continuous flow, leading to water consume of about 100 L/m^2 of plated surface (Grebenyuk *et al.* 1996). This leads to the production of large volumes of wastewaters containing toxic substances like hexavalent and trivalent Cr, Cu, Ni and Zn ions. Metal concentrations in untreated electroplating effluents can be 120 higher than those permissible by law (Volesky 2003).

The effluents from electroplating industries require a pre-treatment step prior to discharge in municipal sewage treatment plants. Most of the techniques for heavy metals removal have drawbacks such as: high capital investments and running costs (reverse osmosis and ion exchange); incomplete metal removal, not capable of meeting the standards (chemical precipitation) (Volesky 2001). The toxicity of the industrial effluents, associated with the increasingly strict regulations introduced by governments, have led to find more efficient and cost-effective treatment technologies. In the last two decades, alternative biomasses have been investigated in order to develop a biological-based technology for detoxification of metal-bearing wastewaters (Wang and Chen 2009).

Among the different types of biological materials, *Saccharomyces cerevisiae* has been recognized as an effective biomass for the treatment of hazardous wastes containing heavy metals, as it was reviewed by Wang and Chen (2006). In particular, flocculent cells of *S. cerevisiae* (flocculation is a characteristic usually found in brewing

yeast) (Soares 2009) can constitute a reliable supply of biomass for large-scale application in bioremediation processes. In this respect, it is well documented that *S. cerevisiae* cells have the ability to remove Ag, Mn, Cd, Co, Cr, Cs, Cu, Ni, Pb, Sr, U and Zn from water solution (Simmons *et al.* 1995; Ferraz and Teixeira 1999; Zouboulis *et al.* 2001; Parvathi and Nagendran 2007; Chen and Wang 2008; Machado *et al.* 2008; Machado *et al.* 2009; Ruta *et al.* 2010). *S. cerevisiae* has the "status" of "generally being recognised as safe" by the US Food and Drug Administration (FDA), which mean that it can be handled by humans without any concern. As a waste product of brewing industries, this biomass can be obtained in large quantities at very low cost; these are two critical points (availability and price), which should be taken into account when seeking a biomass for a biosorption-based technology (Tsezos 2001).

The accumulation of heavy metals by inactivated yeast cells (bisosorption) is influenced by several factors such as the presence of other cations and anions in solution. In addition, pH and the presence of organic or inorganic ligands can affect metals speciation (the different physicochemical forms of the metal in solution, which together make up its total concentration) (Florence 1983) and consequently the available metal to be removed by yeast cells.

This work reports the use of a hybrid technology for the treatment of two electroplating effluents in a batch mode. The process developed combines chemical precipitation with a subsequent biotechnological-based process (heat-treated cells of a flocculent brewer's yeast of *S. cerevisiae*). In addition, a chemical speciation approach was used to define rationally the amount of biomass to be applied on the efficient treatment of the effluents with a minimum number of batches. The advantages of using a flocculent *S. cerevisiae*-based biosorbent for obtaining an effective, competitive and low-cost technology for the treatment of wastewaters loaded with heavy metals is discussed.

6.2. Materials and methods

6.2.1. *Strains, media and culture conditions*

In this work, the flocculent brewing strain of *Saccharomyces cerevisiae* National Collection of Yeast Culture (NCYC) 1364 was used.

Additional information about culture maintenance and pre-cultures, and cultures was described previously (Chapter 2, Section 2.2.1).

6.2.2. *Preparation of biomass*

In this work, biomass preparation was performed as previously described (Chapter 4, Section 4.2.2).

6.2.3. *Determination of biomass*

Cell concentration was determined as previously described (Chapter 3, Section 3.2.3).

6.2.4. *Effluents characterization*

Two effluents (A and B) from an electroplating industry of the Metropolitan Area of Oporto, Portugal, were used in this study. To characterize these effluents, pH value, redox potential, heavy metals (Cu^{2+}, Ni^{2+}, Zn^{2+}, total Cr and Cr^{6+}), total inorganic ligands (chlorides, sulphates, inorganic phosphates, nitrates and fluorides) and total organic and inorganic carbon concentrations were determined. Cr^{6+} and

inorganic ligands were analysed according to the Standard Methods (APHA *et al.* 1998). All determinations were done at least in duplicate.

The determination of pH was attained with a conventional glass electrode using a pH meter Orion, model 4210.

Measurements of redox potential were performed using a combined platinum-ring electrode (Metrohm).

In effluent A, fluorides were determined by direct potentiometry, as described in Chapter 4, Section 4.2.4. In the case of effluent B, due to the high amount of Cr and other heavy metals, which act as interferences, fluorides were determined by ion-chromatography. For this purpose, the effluent was pretreated with an excess of sodium bisulphite to reduce Cr^{6+} to Cr^{3+} and then, a strong complexing agent (1,2 – cyclohexanedinitrilotetraacetic acid) (CDTA) (Fluka) was added; the solution was heated at 60 ºC, during 10 minutes. Afterwards, effluent was filtrated through a 0.45 μm pore size membrane and appropriately diluted. The samples were injected into a mobile phase of sodium carbonate 0.9 M (bubbled in nitrogen at 2000 Psi) and carried through an ion exchange column (Dionex AS9-HC), flowing at 1 ml/min, in an ion-chromatograph (Dionex DX-120).

Chlorides were determined by direct potentiometry using a silver-ion electrode (Crison, Barcelona, Spain) assembled to a Crison Basic 20 pH meter. This method can be done without a pre-treatment step for samples containing phosphates, Cr and other heavy metal ions. Barium nitrate and bisulphite buffer 0.4 M, at pH 2, were added. The solution was titrated with a standard $AgNO_3$ solution.

Nitrates, sulphates, inorganic phosphates, TC, IC, total heavy metals and Cr^{6+} concentrations were determined as described in Chapter 4, Section 4.2.4. In the case of effluent B, standard addition method was used for sulphates and inorganic phosphates determinations in order to minimize matrix interference.

6.2.5. *Computer chemical simulations*

All chemical speciation calculations were carried out as described in Chapter 4, Section 4.2.6. For each case, computational simulations were performed, at pH 6.0, considering the total heavy metals and ligands (inorganic ligands quantified in the real effluent and inactivated biomass) concentrations, as well as all the affinity constants between heavy metals and ligands (Ferraz and Teixeira 1999; Martell and Smith 2004; Machado *et al.* 2009) and solubility product constants (Martell and Smith 2004). The complexation parameters between heavy metals (Cu^{2+} or Ni^{2+} or Zn^{2+}) and biomass were determined in this Thesis (Chapter 3).

6.2.6. *Effluents treatment*

The efficiency of using a hybrid technology for the removal of heavy metals from electroplating effluents was tested using two real effluents (A and B). Effluent A, containing Cu, Ni and Zn, was adjusted to pH 6 with NaOH and after agitation the effluent was allowed to stand undisturbed for one night and then filtrated through a 0.45 μm-pore size membrane. Effluent B, containing Cr, Cu, Ni and Zn, was treated with an excess of sodium bisulphite to assure the reduction of Cr^{6+} to Cr^{3+}. The efficiency of this process was controlled by the determination of Cr^{6+}, as described in Chapter 4, Section 4.2.4. Then, the effluent was diluted four times and the pH was adjusted to 6 with NaOH; the effluent was allowed to stand undisturbed for one night and then filtrated through a 0.45 μm-pore size membrane. Subsequently, washed cells with a concentration near 18 g dry weight/l were added to the effluents and shaken in 500 ml plastic flasks at 150 rpm and 25 ºC. After 30 minutes of contact between the biomass and the effluent, cells were removed and the supernatant added to new yeast biomass, in a subsequent batch. Kinetics studies previously performed (Chapter 3) have shown that a contact time of 30 minutes was enough to reach the equilibrium between dead cells and metals solution.

Control experiments were done incubating the effluents in the absence of yeast cells under the same conditions described above; these controls have shown that no appreciable amount of metals (less than 1%, for all metals in both effluents) was adsorbed to the plastic flasks. Heavy metals concentrations were determined as described above.

6.2.7. *Reproducibility of the results*

All experiments were performed in duplicate and repeated, at least, three times (n = 6). In Figure 6.2, vertical error bars representing standard deviation were not shown for pictorial clarity.

6.3. Results

6.3.1. *Characterization of the industrial effluents*

Real effluents were collected from a Portuguese electroplating unit, which is devoted to the production of bathroom accessories. Effluents were sampled from two independent tanks, which receive the wastewaters from the pickling (both effluents receive wastewaters from these lines and contain Cu and Zn) and Ni (effluent A) or Cr-Ni (effluent B) electroplating lines.

Firstly, physicochemical characterization of the effluents was performed (Table 6.1).

Table 6.1. Physicochemical parameters of raw electroplating effluents and after alkaline treatment (pH adjustment to 6.0). The level of contaminants are compared to the Environmental Protection Agency (US-EPA, 1984) and the Portuguese (Decreto-lei 236/98) wastewater limit discharge criteria.

		Wastewater limit discharge criteria		**Industrial effluent**			
		US-EPA	**Portuguese law**	**A**		**B**	
pH	(units)		6.0-9.0	2.1	6.0	2.3	6.0[#]
E_h	(mV)			474		593	
TC	(mg/l)			8.3		11.5	
TOC	(mg/l)			7.37		10.72	
IC	(mg/l)			0.9		0.8	
Cl^-	(mg/l)			114.0		182.0	
F^-	(mg/l)			2.3		1.1	
NO_3^-	(mg/l)		50	5.2		7.4	
SO_4^{2-}	(mg/l)		2000	350		200	149
PO_4^{3-}	(mg/l)			33		0.13	
Cu^{2+}							
	(mg/l)	3.38	1.0	19.6	3.7	10.5	1.5
	(μM)	53.1	16	308	58	165	24
Ni^{2+}							
	(mg/l)	3.98	2.0	22	21	75	21
	(μM)	67.8	34	366	358	1278	353
Zn^{2+}							
	(mg/l)	2.61		25	17	5.7	1.0
	(μM)	39.9		381	266	87	15
Total Cr							
	(mg/l)	2.77	2.0	1.1	0.32	97	15
	(μM)	53.3	38	21	6	1865	288
Cr^{6+}							
	(mg/l)		0.1	<0.06	<0.06	75	<0.06
	(μM)		2	<1	<1	1442	<1

TC: total carbon. **TOC:** total organic carbon. **IC:** inorganic carbon.
[#] After Cr (VI) reduction with bisulphide, dilution (4 times) and pH adjustment to 6.0.

The Table indicates that raw effluent A, at pH 2.1, presents Cu, Ni and Zn concentrations above the limits of discharge allowed by the US-Environmental Protection Agency (US-EPA 1984) and by Portuguese law (Decreto-lei 236/98). Raw effluent B, at pH 2.3, presents Cr, mainly (77%) in the form of Cr^{6+}, Cu and Ni above the limits of discharge. On the other hand, Table 6.1 shows that no

appreciable amount of organic matter is present in both effluents and the concentration of inorganic ligands are below the legal limit of discharge under Portuguese law.

6.3.2. *Treatment of real effluents: the hybrid technology*

A hybrid technology, combining chemical precipitation with a subsequent biotechnological-based process (heat-treated cells of a flocculent brewer's yeast of *S. cerevisiae*), at pH 6.0, was developed for the treatment of real electroplating effluents containing multi-metals.

Hence, the hybrid process started with the alkaline treatment (adjustment to pH 6). In the case of effluent B, reduction of Cr^{6+} to Cr^{3+} was performed, using sodium bisulphite (a reagent widely used in the electroplating industries for this purpose) with effluent pH 2.3, according to the following reaction:

$$2HCrO_4^-{}_{(aq)} + 5H^+{}_{(aq)} + 3HSO_3^-{}_{(aq)} \rightarrow 2Cr^{3+}{}_{(aq)} + 5H_2O_{(l)} + 3SO_4^{2-}{}_{(aq)} \quad [6\text{-}1]$$

After Cr reduction (redox potential = 220 mV) and before metals accumulation by yeast cells, effluent B was diluted four times and the pH of solution was raised up to 6.0 (Table 6.1). In a similar way, the pH of effluent A was also raised up to 6.0 (Table 6.1). As a consequence of this increase of pH, a significant amount (81%) of Cu was removed from effluent A by chemical precipitation. In the case of effluent B, about 40% of Cr^{3+} and Cu were removed from solution due to the raise of pH (Table 6.1). Chemical speciation studies at pH 6.0 predicted that zinc should be totally soluble mostly as Zn^{2+} (56% of total Zn for effluent A and 74% for effluent B) and $ZnSO_4$ (42% of total Zn for effluent A and 25% for effluent B). Even though no precipitation of Zn was predicted at pH 6.0 by chemical speciation studies, a reduction of about 30% of the total amount of soluble Zn, initially present

in both effluents, was also attained. This fact was probably due to coprecipitation of Zn with Cu (effluents A and B) and Cr (effluent B).

The choice of pH 6 for this hybrid technology was due to the following reasons: (i) the consume of hydroxide necessary to raise the pH of the effluent is not significant; (ii) pH 6 corresponds to the minimum pH value of wastewater discharge according to the Portuguese law (Table 6.1); (iii) at this pH value, if effluents contain large amounts of Cr^{3+} and Cu, part of this precipitates as metal hydroxides (Martell and Smith 2004), as shown above (Table 6.1), and thus more biomass will be available for removing Ni and Zn by biosorption; (iv) at this pH, the efficiency of the biotechnological-based process should be high because the main chemical binding groups of the yeast cells surface (carboxyl, phosphate, sulfydryl, hydroxyl and nitrogen-containing groups), are already totally (carboxylic acids) or partially (phosphate and amine groups) deprotonated (Martell and Smith 2004).

In order to predict the efficiency of the biosorption process for those metals having concentrations in the effluents above the limits of discharge, the theoretical amounts of metal ions accumulated by yeast cells were chemically simulated using an iterative process. For this purpose, the MINEQL+ software (Schecher and McAvoy 2003) was used. The program was run with a fixed concentration of biomass up to 18 g/l, at pH 6.0, assuming the chemical composition of the effluents (Table 6.1). Chemical simulations have shown that 18 g/l of biomass should be efficient for the treatment of effluents A and B (diluted four times) at pH 6.0. Figure 6.1 shows species distribution of metals in solution (free metal ions plus metal ions complexed with sulphates) and adsorbed to yeast cells, after the first batch, when 18 g/l of biomass is simulated. Cr, in effluent A, and Cu and Zn, in effluent B, were considered in the theoretical simulations, but not shown in Figure 6.1, because concentration values in the effluents, at pH 6.0, were close or below the discharge limits (Table 6.1). These simulations predict that the major amount of metals (> 89%) should be removed (accumulated by the yeast cells) after the first batch. Taking

into account the chemical simulation obtained above, the biotechnological-based treatments of both industrial effluents, after chemical precipitation, were carried out using 18 g/l of biomass; under these conditions, the molar ratio of [Biomass]/∑ [M] was 10 for both effluents.

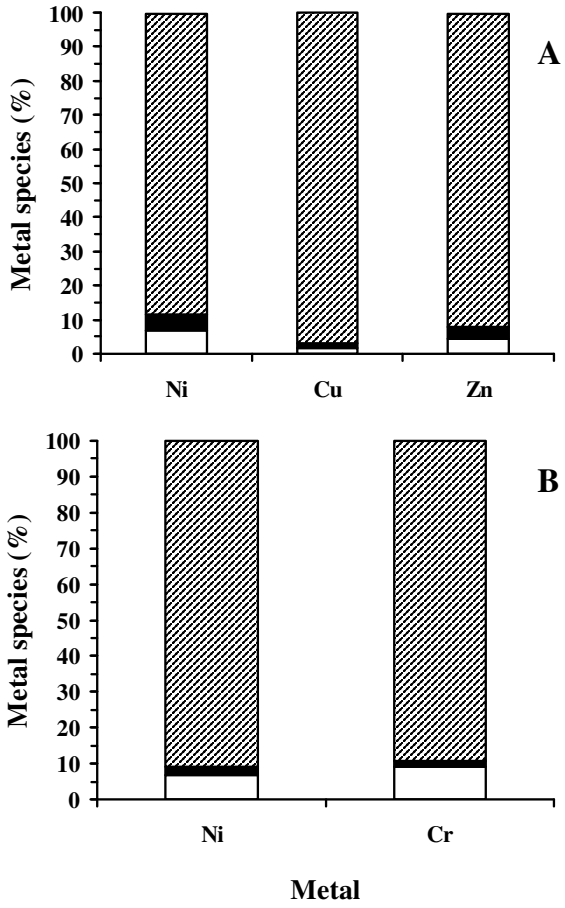

Figure 6.1. Chemical simulation of the influence of biomass on the metal species distribution after the first batch. 18 g/l dry weight yeast cells of *S. cerevisiae* were used. **A** – industrial effluent A; **B** – industrial effluent B. Metals associated with inactivated yeast cells (dashed bar); metals associated with sulphate (black bar); free metal ions (white bar). Chemical simulations were performed with a chemical equilibrium computer program (MINEQL+).

After a chemical treatment (pH adjustment to 6.0), a serial batch reactor, using new biomass in each batch, was implemented. Metal final concentrations and cumulative percentages of metal removals obtained, after each batch, are present in

Table 6.2 and Figure 6.2, respectively; theoretical simulation of cumulative percentages of metals removals (only for those metals, which concentrations in the effluents at pH 6.0 were above the limits of discharge) were also calculated and included in the Figure. Figure 6.2 shows that after the first batch, the highest removal percentages were obtained for Cu (93% for both effluents); thus, Cu concentration meets the quality criteria according to the US-EPA and Portuguese law for both effluents.

For effluent A, Ni and Zn removals were 57 and 59%, respectively; in the case of effluent B, Cr and Ni removals were 67 and 59%, respectively. In the subsequent batch, metal concentrations in solution were reduced and Cr concentration, in effluent B, reached the quality criteria, according to both laws. After the third batch, both effluents were treated, i.e., metals concentrations in solution meet the quality criteria according US-EPA and Portuguese laws. Ni and Zn concentrations, in effluent A, lowered to 2.4 and 1.1 mg/l, respectively (Table 6.2). These values corresponded to Ni and Zn cumulative removals of 89 and 94%, respectively, which are in good agreement with the theoretical simulations (Figure 6.2). For effluent B, a similar behaviour was observed; total concentrations of Cr and Ni decreased to 1.2 and 2.2 mg/l, after the third batch (Table 6.2). These values corresponded to total Cr and Ni cumulative removals of 92 and 90%, respectively, and are also in good agreement with the theoretical predictions (Figure 6.2).

Table 6.2. Bioremediation of industrial effluents using *S. cerevisiae* NCYC 1364, in a batch mode reaction. The level of the metals reached is compared with the Environmental Protection Agency (US-EPA 1984) and the Portuguese (Decreto-lei 236/98) wastewater limit discharge criteria.

		Metal concentration (mg/l)[#]					
		Limit discharge criteria		Effluent at pH 6.0	After 1st batch	After 2nd batch	After 3rd batch
	Effluent[##]	US-EPA	Portuguese law				
Ni^{2+}	A	3.98	2.0	21 ± 1	9± 1	4 ± 1	2.4 ± 0.4
	B			21 ± 1	8.6 ± 0.7	3.5 ± 0.5	2.2 ± 0.4
Zn^{2+}	A	2.61	NS	17± 1	7 ± 2	3 ± 1	1.1 ± 0.4
	B			1.0 ± 0.1	ND	ND	ND
Cu^{2+}	A	3.38	1.0	3.7 ± 0.2	0.25 ± 0.04	ND	ND
	B			1.5 ± 0.4	0.1 ± 0.1	ND	ND
Total Cr	A	2.77	2.0	0.32 ± 0.01	ND	ND	ND
	B			15 ± 3	5 ± 1	1.9 ± 0.2	1.2 ± 0.2
Cr^{6+}	A	NS	0.1	<0.06	ND	ND	ND
	B			<0.06	ND	ND	ND

ND: not determined; the metal concentrations were below the legal limit of discharge. NS: not specified in the law.
#Mean and standard deviation of three independent experiments performed in duplicate *(n=6)*.
Effluent A: after pH adjustment to 6.0. Effluent B: after Cr (VI) reduction with bisulphide, dilution (4 times) and pH adjustment to 6.0.

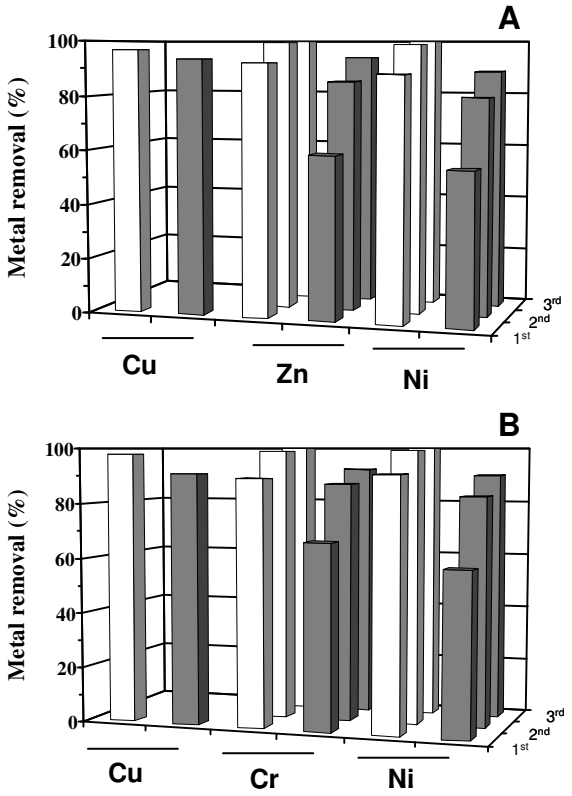

Figure 6.2. Comparison of theoretical and experimental metals removal from electroplating effluents by *S. cerevisiae* NCYC 1364 through a series of sequential batches in bench scale experiments. A – industrial effluent A; B – industrial effluent B. Theoretical values: white bar; experimental values of heavy metals removal: dark bar. Theoretical calculations were performed with a chemical equilibrium computer program, as described in Section 6.2.5. Experimental assays were carried out with 18 g dry weight/l of biomass. Values are the mean of two replicates of three independent experiments (n = 6).

6.4. Discussion

In recent years, different types of biomass (such as *Sargassum wightii, Aspergillus niger, Aspergillus sydoni, Penicillium janthinellum, Ulva reticulata, Geobacillus thermodenitrificans*) have been used for the treatment of wastewaters containing heavy metals (Vijayaraghavan *et al.* 2006; Kumar *et al.* 2008; Vijayaraghavan 2008; Prigione *et al.* 2009; Chatterjee *et al.* 2010). However, the treatment of real effluents using *S. cerevisiae* has rarely been reported in the literature (Gadd 2009). The removal of heavy

metals is usually performed with a mono element in very simple solutions, usually in almost absence of anionic ligands (Simmons *et al.* 1995; Zouboulis *et al.* 2001; Matis *et al.* 2003; Machado *et al.* 2008). Real effluents are, as a rule, much more difficult to treat due to the presence of multi-metals and multi-ligands in solution. In addition, the use of real effluents instead of synthetic (simulated) ones constitutes a challenge in order to test and validate the usefulness of the use of microbial biomass in the bioremediation of heavy metals.

Heavy metals accumulation by dead yeast biomass can be due to one or more of the following mechanisms: adsorption, precipitation or complexation by yeast cell surface structure (Blackwell *et al.* 1995). Most likely, carboxyl, amino, hydroxyl and amide groups of protein and carbohydrate fractions (most likely of mannoproteins, glucans and chitin) of the cell wall are involved in the yeast metal uptake (Blackwell *et al.* 1995; Machado *et al.* 2009). Cr, Cu, Ni and Zn are the metal ions most widely found in the electroplating effluents and the affinity between biomass used in this work and these metal ions follows the following order: Cu >> Cr ≅ Zn > Ni (Ferraz and Teixeira 1999; Machado *et al.* 2009). Effluents containing multi-elements are more complex because metal ions compete for binding sites; as a consequence, displacement of one metal species by another one, which has higher affinity for biomass binding sites, can occur (Brady *et al.* 1994). Thus, for bioremediation of effluents containing multi-elements, the amount of biomass ([Biomass]) when compared with the total amount of metals ($\sum$ [M]) should be in excess in order to have sufficient available biomass sites for all metals accumulation, including those with lower affinity for biomass. As it was discussed in Chapter 5, owing to the lower affinity of biomass to Ni (log K = 3.34, at pH 6.0), the efficiency of bioremediation of effluents containing Ni among other metals (Cr, Cu and Zn) is conditioned by Ni affinity. For an effective biorremediation, Ni-loaded effluents should not have high concentrations of Ni. For these reason, effluent B was diluted about four times before any biosorption step has been performed.

After alkaline treatment (adjustment to pH 6), the concentration of Cu in effluents A and B was of the same order of magnitude (Table 6.1). This is due to the fact that the concentration of a soluble metal ion in equilibrium with the corresponding insoluble metal hydroxide, $M(OH)_x$, at a fixed pH value is a constant value: $[M^{x+}_{(aq.)}] = Kps/[OH^-_{(aq.)}]^x$; Kps is the solubility product and is a constant value and the concentration of hydroxide, at a fixed pH value, is also a constant value. Owing to the high insolubility of Cr^{3+} and Cu^{2+} hydroxides (Martell and Smith 2004), this first step of effluents remediation (alkaline treatment) allows to reduce concentrations of Cr^{3+} and/or Cu^{2+} from the real effluents to very low concentrations whatever the initial concentration of these two metals present in the wastewaters.

As it can be seen in Table 6.1, after the alkaline treatment, effluents are suitable for implementing the biotechnological-based step. This aspect is particularly important when we have multi-elements in the effluent competing to biomass, which frequently occurs in the electroplating effluents. In fact, heavy metals removal by yeast cells is pH dependent. At pH 2.0, metal cations uptake by yeast cells is practically not observable, increasing with the pH raising, being usually performed at pH values between 5 and 6 (Brady *et al.* 1994; Ferraz and Teixeira 1999; Özer and Özer 2003).

In the present work, brewing flocculent yeast cells were used in the biotechnological-based step. Yeast flocculation is a reversible process wherein the individual cells aggregate into multicellular masses, called flocs (composed by thousands or even millions of cells), which sediment rapidly in the medium in which they are suspended (Soares 2009). Flocculent yeasts have in their cell wall a specific lectin-like protein which allow to interact with the carbohydrate residues of the walls of neighbouring cells and establish a flocculent bond; in this process Ca^{2+} seems to ensure the correct conformation of the lectins (Soares 2009). Flocculent cells of *S. cerevisiae* have a higher ability of Cu^{2+} accumulation than the non-flocculent cells

(Soares *et al.* 2002a) most likely due to the fact that heavy metals can also occupy lectin Ca^{2+} binding sites. In Chapter 3, we have shown that yeast cells, inactivated at 45 ºC, maintained their flocculent properties, when compared with live cells, in industrial effluents and in a wide range of synthetic effluents containing various metals. The yeast strain used is able to form flocs of macroscopic size, being achieved a sedimentation > 95% of cells within 5 minutes (Machado *et al.* 2008), as it was demonstrated in Chapter 2. In addition, yeast-killed cells were able to accumulate higher quantities of Ni and Zn than live yeast cells, at pH 6.0 (Chapter 3). Since the removal of heavy metals and cell separation are simultaneously achieved when flocculent biomass is used, the employment of this type of cells looks a promising alternative for the bioremediation of metal-loaded industrial effluents. Moreover, the natural aggregation properties of flocculent yeast cells allows the use of the sedimentation tanks, already available in the wastewater treatment plants of the major electroplating industries for chemical precipitation of effluents. The process can be feasibly engineered on a large scale and avoids the capital investment when changing from the conventional (chemical precipitation) to the process proposed. Moreover, stirred batch tank reactor has the advantages of design simplicity, stability of operation, low operational and maintenance costs. Together, these are important points when seeking a competitive and inexpensive technology. In addition, if desired, flocculent yeast cells can also be used in continuous mode, in columns, without the risk of biomass washout.

As it can be seen in Table 6.2, after the first batch, the concentrations of Cu were below the limits of discharge criteria (US-EPA 1984; Decreto-lei 236/98) in both effluents. This fact was due to the high insolubility of Cu hydroxide, which allows the removal of a large amount of Cu from solution by chemical precipitation at pH 6.0. Additionally, the high affinity of yeast cells for Cu removes efficiently the remained concentration of Cu in solution. These results demonstrate that, for effluents containing only Cu, the application of this hybrid technology at pH 6.0 is

efficient, after the first batch, whatever the initial concentration of Cu present in the effluent.

The percentages of experimental Cu removals found (93% for both effluents) are in very good agreement with the theoretical simulations, for which Cu removals of 97 and 98% were predicted for effluents A and B, respectively (Figure 6.2). On the other hand, the percentages of experimental removals found for the other metals (Cr, Ni and Zn) in both effluents were lower than the theoretical simulations (Figure 6.2). However, the differences between theoretical and experimental values were attenuated with the number of cycles; after the third cycle, for effluent A, the differences for Ni and Zn were 11 and 6%, respectively, whereas for effluent B, the differences for Ni and Cr were 10 and 8%, respectively. These differences can be due to several factors: (i) an overestimation of the values of metals adsorbed to yeast cells, calculated by the theoretical simulation, as a consequence of the stability constants between these metals and inorganic ligands, used in the simulations, were not determined in the experimental conditions of the effluents (e.g. ionic strength and temperature); (ii) the affinity constants between metals and biomass (Ferraz and Teixeira 1999; Machado *et al.* 2009) were determined using the Langmuir model; this model, which is mostly widely used by the generality of the authors, assumes that all sites of the biomass have the same affinity and does not describe the heterogeneity of the yeast cells and the effects of ionic strength and metal mixtures (Lodeiro *et al.* 2005; Herrero *et al.* 2006); (iii) complexation of metals by organic matter present in the real effluents and soluble organic matter leached from the microorganisms; the release of metal binding agents into solution from the cytoplasm of yeast cells, as a consequence of the damaging of cell membrane during the drying process (Chapter 3), complex metals and thus reduce the efficiency of bioremediation process (Soares *et al.* 2002b; Soares *et al.* 2003). The values of TOC in both effluents were $\leq$ 11 mg/l (Table 6.1), which indicates that effluents contain low amount of organic matter. However, it is important to point out that minor amounts of strong complexing agents, such as ethylenediaminetetracetic (EDTA) or nitrilotriacetic acid (NTA) and

citric acid can be present in the effluents due to their use in the soak cleanings and pickling steps (Schlesinger 2004), respectively. These compounds complex strongly with these metal ions (Martell and Smith 2004) and their presence, even in minor amounts, can raise the amount of soluble metal ions not available to be accumulated by yeast cells.

Heat-treated flocculent yeast cells have shown to be very effective in the metals removal from industrial effluents. After the third batch, both effluents were treated (Table 6.2), with removals of Ni, Cu, Cr and Zn $\geq$ 89, 91, 92 and 94%, respectively. As was reported above, only few papers have been devoted to the treatment of real effluents using yeast biomass. Duncan and co-workers used *S. cerevisiae* cells, in a batch mode (Stoll and Duncan 1996), immobilized cells in continuous–flow stirred bioreactors (Stoll and Duncan 1997) or in fixed bed-columns (Zhao and Duncan 1998); however, no complete treatment of the effluents was achieved. More recently, the difficulty in the treatment of real effluents, due to its complex composition, was shown (Cojocaru *et al.* 2009); the treatment of a real wastewater containing Cu was not successful, being the % of metal removal in the industrial effluent lower than in aqueous solution (Cojocaru *et al.* 2009). Conversely, the hybrid methodology developed showed to be an effective process on the treatment of real effluents containing multielements.

An important point related with the use of biomass to treat industrial wastewaters containing heavy metals is linked to the fate of contaminated biomass after effluent treatment. Metal-loaded biomass can be strongly reduced by incineration. Subsequently, ashes can be acid digested and the metals selectively recovered. This strategy was successfully applied in Chapters 8 and 9.

6.4. Conclusions

In this work, a hybrid technology, which combines chemical precipitation with subsequent biosorption, at pH 6.0, was developed for removing multi-elements (Cr, Cu, Ni and Zn) from real electroplating effluents. For effluents containing Cr^{3+} and/or Cu, chemical precipitation followed by two biosorption batch steps will be enough to reduce metal(s) concentration(s) to values below the legal limits of discharge, whatever the concentration of Cr^{3+} and/or Cu present in the original effluent. On the other hand, effluents containing multi-elements (Cr, Cu, Ni and Zn), the amount of biomass ([Biomass]), when compared with the total amount of metals to be biosorbed ($\sum$([M]) should be in excess. The use of 18 g/l of dried yeast cells of *S. cerevisiae* removed efficiently 21 and 17 mg/l of Ni and Zn to the limits of discharge allowed by an International Agency (EPA) and Portuguese law, after the implementation of a sequential serial reactor (three batches) at pH 6.0.

In this work, it has been demonstrated that the use of brewing flocculent yeast cells is a new and promissory approach to the bioremediation of industrial effluents, since it combines an inexpensive process for obtaining biomass, an eco-friendly process of cell separation and an efficient removal of heavy metals. Together, these are critical issues when looking for an effective and low cost technology.

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Chapter 7

Removal of chromium, copper and nickel from an electroplating effluent using a flocculent brewer's yeast strain of *Saccharomyces cerevisiae* [6]

Abstract

The release of heavy metals in aquatic systems due to the discharge of industrial wastewaters is a matter of environmental concern. Heat-inactivated cells of a flocculent strain of *S. cerevisiae* were used in the bioremediation, in a batch mode, of a real electroplating effluent containing Cu, Ni and Cr. In this approach, no previous reduction of Cr^{6+} to Cr^{3+} was required. Cr^{6+} was selectively removed (98%) by yeast biomass at pH 2.3. At this pH, Cr^{6+} is mainly in the form of $HCrO_4^-$ and yeast surface is surrounded by H^+ ions, which enhance the Cr^{6+} interaction with biomass binding sites by electrostatic forces. Subsequently, pH of the effluent was raised up to 6.0; this pH maximizes the efficiency of cations removal since at this pH the main binding groups of yeast cells are totally or partially deprotonated. The passage of effluent through a series of sequential batches, at pH 6.0, allowed, after the third batch, the removal of Cu^{2+}, Ni^{2+},Cr total and Cr^{6+} in the effluent to values below the legal limit of discharge.

The strategy proposed in the present work can be used in plants for the treatment of heavy metals rich industrial effluents containing simultaneously Cr^{6+} and Cr^{3+}.

[6] Water, Air, & Soil Pollution (DOI 10.1007/s11270-010-0332-1)

7.1. Introduction

Electroplating industries generate large amounts of effluents containing heavy metals, such as Cu, Cr, Ni and Zn. Owing to heavy metal toxicity, electroplating wastewaters require a previous treatment before being discharged in surface waters or sending to a municipal sewage treatment plant.

Notwithstanding an increased interest has been observed, in the last years, in the use of different biological materials for the removal of heavy metals, a gap still exists between the proposal of this materials and their application to real effluents (Gadd 2009). The studies found in the literature are usually carried out with a single element in very simple solutions (simulated wastewaters); real effluents are, as a rule, much more complex to treat.

In this work, the efficiency of heat-killed cells of a brewing strain of *S. cerevisiae* to remove copper, nickel and chromium, from a real electroplating effluent, in a batch mode, was evaluated. In addition, the removal of Cr^{6+} without a previous reduction to Cr^{3+} was also assessed. As far as we know, this is the first work, which selectively removes Cr^{6+} and Cr^{3+}, without a previous reduction step, wherein total Cr level, in treated effluent, reach the stipulated effluent discharge criteria.

7.2. Materials and methods

7.2.1. *Strains, media and culture conditions*

The flocculent brewing strain of *Saccharomyces cerevisiae* National Collection of Yeast Culture (NCYC) 1364 was used.

Additional information about culture maintenance and pre-cultures, and cultures was described previously (Chapter 2, Section 2.2.1).

7.2.2. *Preparation of cell suspensions*

In this work, biomass preparation was performed as previously described (Chapter 4, Section 4.2.2).

7.2.3. *Metals quantification*

An effluent from an electroplating industry from the Metropolitan Area of Porto, Portugal, was used. This effluent corresponded to effluent B, previously used in Chapter 6. Total heavy metals and Cr^{6+} concentrations were determined as previously described in Chapter 4, Section 4.2.4.

7.2.4. *Theoretical simulation of metals removal*

All chemical speciation calculations were carried out as described in Chapter 4, Section 4.2.6. Computational simulations were performed considering (i) at pH 2.3, the total concentration of Cr^{6+} in the industrial effluent and (ii) at pH 6.0, the total concentrations of Cr^{3+}, Cu^{2+} and Ni^{2+} present after the first batch. At both pH values, affinity constants and q_{max} between metals and biomass were also taken into account.

At pH 2.3, affinity constants and q_{max} between Cr^{6+} and biomass assumed in the computational calculations were described by Özer and Özer (2003). At pH 6.0, affinity constants and q_{max} used in the computational calculations between Cr^{3+} and biomass were previously described by Ferraz and Teixeira (1999). The complexation

parameters between Cu^{2+} or Ni^{2+} and biomass were determined in this Thesis (Chapter 3).

7.2.5. *Bioremediation of effluent in a multi-batch reaction system*

Washed biomass, with a concentration near 18 g dry weight/l, was added to the industrial effluent (diluted 4 times), at an initial pH 2.3. The suspension was shaken, at 150 rpm, at 25ºC, in 500 ml plastic flasks. After 30 minutes of contact between the biomass with the effluent, cells were removed. Subsequently, the pH of suspension was adjusted to 6.0 with NaOH; the suspension was allowed to stand undisturbed for one night. New yeast biomass was added to the effluent and mixed as described above, during 30 minutes. Then, cells were removed and the supernatant added to new yeast biomass, in the subsequent batches. Kinetic studies, previously performed (Chapter 3, Section 3.3.1), have shown that a contact time of 30 minutes was enough to reach the equilibrium between cells and metals solution. A small modification of pH (lesser than 0.5 units) was observed throughout the three batches at pH 6.0. Control experiments were done incubating the effluent in the absence of biomass, in the same conditions described above; no appreciable amount of metals (less than 1%) was adsorbed to the plastic flasks.

7.3. Results and discussion

The effluent was collected from a Portuguese electroplating unit, which is dedicated to the production of bathroom accessories. The sample was taken from a tank which receives the wastewaters from the pickling (contain copper and zinc) and chromium-nickel electroplating lines. As it can be seen in Table 7.1, the industrial effluent, diluted 4 times, at an initial pH 2.3, contains chromium, mainly (75%) in the form of Cr^{6+}, and nickel above the limits of discharges allowed by the US-

Environmental Protection Agency (US-EPA 1984) 1984) and the Portuguese law (Decreto-lei 236/98); copper is also above the limits of discharge, according to Portuguese effluent discharge criteria.

While Cr^{3+}, in trace amount, is an essential nutrient (in humans, it is required for sugar and lipid metabolism), Cr^{6+} is a known poison, acting on live cells as mutagen and carcinogen (Mertz 1993; Pechova and Pavlata 2007); in addition, it is more soluble and consequently, more mobile than Cr^{3+} (Gomez and Callao 2006). Due to the high toxicity of Cr^{6+}, low values of limits of discharge are associated (0.1 mg/l) (Table 7.1).

A common process in the treatment of industrial effluents containing Cr^{6+} is its reduction to Cr^{3+} with a sulphuric acid solution of bisulphite. In the present work, a new approach was developed, without any reduction process; it consisted in two sequential steps, using heat-inactivated cells of *S. cerevisiae*: the first step was carried out at near pH 2, in order to selectively remove Cr^{6+}; afterwards (second step), the initial pH of the effluent was raised up to 6.0 and subsequent batch series were used in order to remove the cations: Cr^{3+}, Cu^{2+} and Ni^{2+}.

Chemical speciation calculations, using a computational program, performed at fixed pH values (2.3 or 6.0) allowed predicting the amount of Cr^{6+} or Cr^{3+}, Cu^{2+} and Ni^{2+} associated with the biomass, respectively. By an iterative process, it was found that 18 g/l of heat-killed cells of *S. cerevisiae* NCYC 1364 would be enough to treat the effluent (see Chapter 6, Section 6.3.2).

The value of pH 2.3 for Cr^{6+} removal was chosen due to two reasons: i) *S. cerevisiae* cells show a high Cr^{6+} uptake at very acidic conditions (pH 2-2.5) ii) this was the initial pH of the effluent and thus, no modification was required. The choice of pH about 6.0 for removing the further metal ions [(Cr^{3+},Cu^{2+}), and Ni^{2+}] was due to the fact that (i) at this pH, the efficiency of biomass to adsorb metal ions is high

Table 7.1. Bioremediation of an electroplating effluent using *S. cerevisiae* NCYC 1364, in a batch mode reaction.

	Metal concentration (mg/l)[a]						
	Limit discharge criteria		Industrial effluent[b]	After 1st batch[c]	After 2nd batch	After 3rd batch	After 4th batch
	US-EPA	Portuguese law					
Ni(II)	3.98	2.0	23 ± 2	22± 2	11 ± 1	5 ± 1	3 ± 1
Zn(II)	2.61	NS	1.4 ± 0.2	ND	ND	ND	ND
Cu(II)	3.38	1.0	2.57 ± 0.04	2.32 ± 0.03	0.57 ± 0.08	0.25 ± 0.04	0.19 ± 0.04
Total Cr	2.77	2.0	24 ± 3	9± 2	3.7± 0.8	2.4± 0.6	1.7± 0.4
Cr(VI)	NS	0.1	18 ± 1	0.3± 0.2	0.5± 0.5	0.1± 0.1	<0.06

The level of metals reached are compared to the Environmental Protection Agency (US-EPA 1984) and the Portuguese (Decreto-lei 236/98) wastewater limit discharge criteria.
ND: not determined; the metal concentrations were below the legal limit of discharge. NS: not specified in the law.
a)Mean and standard deviation of two independent experiments performed in duplicate *(n=4)*.
b)Heavy metals profile of an electroplating effluent, diluted 4 times, at pH 2.3.
c)Effluent composition after the first batch at pH 2.3. The subsequent batch series was carried out at pH 6.0.

because the main chemical binding groups of the biomass surfaces are already totally (sulphonic and carboxylic acids) or partially (phosphate and amine groups) deprotonated (Martell and Smith 2004) and (ii) the consumption of hydroxide necessary to raise the pH of the effluent is not significant.

Theoretical calculations predicted that after one batch, at initial pH 2.3, about 97% of Cr^{6+} should be removed. As it can be observed in Table 7.1, yeast biomass was very efficient in Cr^{6+} biosorption, remaining in solution 0.3 mg/l, which corresponded to a removal of 98%; this result shows that experimental value fitted quite well with the theoretical prevision. Thus, at this initial pH value, Cr^{6+} was selectively accumulated (Figure 7.1) and the results point out that the remaining Cr, which is mainly in the form of Cr^{3+} (Table 7.1) can be possibly removed at pH 6.0.

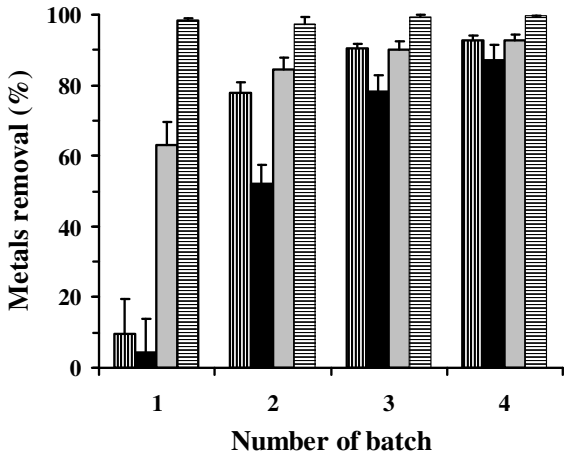

Figure 7.1. Evolution of the percentage of metals removal by heat-inactivated cells of *Saccharomyces cerevisiae* NCYC 1364 through a series of sequential batches. Metals: copper (bar with vertical lines); nickel (dark bar); total chromium (grey bar); chromium (VI) (bar with horizontal lines). Batch assays were carried out with 18 g dry weight/l of biomass, at pH 2.3 (first batch) or pH 6.0 (subsequent batch series). Values are the mean of two replicates of two independent experiments; standard deviations are presented (n = 4).

This fact is well understood because pH influences both metal binding sites of the biosorbent and chromium chemistry in solution. At low pH (pH < 5), the dominant species of Cr^{6+} is $HCrO_4^-$ (Figure 7.2).

On the other hand, the characterization of biosorbents surface by infrared spectroscopy suggested the involvement of carboxyl and amino groups, among others, in the metals removal (Chapter 3). At very low pH values (pH < 3), these groups are protonated. As a consequence, the surface of biosorbent will be surrounded by H^+ ions (Parvathi and Nagendran 2007), which enhance the Cr^{6+} interaction with binding sites of the biosorbent due to electrostatic forces (Özer and Özer 2003). All these facts explain the highest Cr^{6+} removal efficiency attained (Table 7.1).

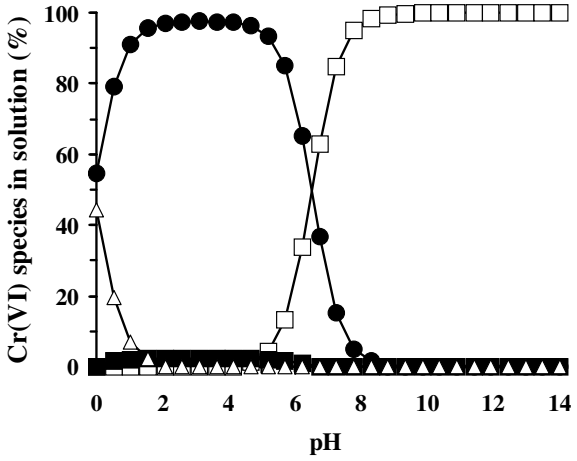

Figure 7.2. Species distribution diagram of 3.5 x 10^{-4} mol/l of Cr(VI) at different pH values. The different species of Cr(VI) in solution were calculated with a chemical equilibrium computer program (MINEQL+): CrO_4^{2-} (□), $Cr_2O_7^{2-}$ (■), $HCrO_4^-$ (●), H_2CrO_4 (Δ).

Additionally, placing the effluent at pH 6.0, allows, maximizing the efficiency of metal cations removal by yeast cells because the main chemical binding groups of the biomass surfaces are already totally or partially deprotonated. In fact, cations removal by yeast cells is pH dependent. At pH 2.0, metal [Cr^{3+},Cu^{2+} and Ni^{2+}] cations accumulation by yeast biomass was practically undetectable (Table 7.1 and Figure 7.1). Optimal pH for Cu(II), Ni(II) and Cr(III) removal by *S. cerevisiae* yeast cells is in the pH range 5-6 (Brady and Duncan 1994; Ferraz and Teixeira 1999; Özer and Özer 2003).

After Cr^{6+} removal and increasing the initial pH of the effluent up to 6.0, the new biomass batch leads to a removal of 52% of Ni^{2+} and 78% of Cu^{2+} (Figure 7.1);

at this stage, Cu^{2+} concentration in solution was below the limit of effluent discharge (1.0 mg/l) according to the Portuguese law (Table 7.1). After the fourth batch, Ni^{2+} and total Cr concentrations in solution were 3 and 1.7 mg/l, respectively (Table 7.1), which met the quality criteria for industrial effluents discharge, according to the US-EPA (for both metals) and the Portuguese law (for total chromium); these values corresponded to a removal of 87 and 93% of Ni^{2+} and total Cr, respectively (Figure 7.1). In addition, after the fourth batch, Cr^{6+} in solution was lower than 0.06 mg/l, which was below the stipulated criteria of effluent discharge according to the Portuguese law (Table 7.1). On the contrary to the works described in the literature using *S. cerevisiae* cells in a batch mode (Cojocaru *et al.* 2009), in fixed bed-columns with immobilized cells (Zhao and Duncan 1998) or using filamentous fungi (Prigione *et al.* 2009), the process developed in this paper showed efficient heavy metals removal from complex industrial effluents and allowed achieving the treatment of the effluent.

7.4. Conclusions

The procedure developed in the present work showed to be an efficient process for metals bioremediation of a real and complex (multi-element) effluent, without a pre-treatment process of Cr^{6+} reducing. Using heat-inactivated cells of *S. cerevisiae*, after the first batch at an initial pH 2.3, 98% of Cr^{6+} was removed. Then, the amount of Ni^{2+}, Cr^{6+},Cu^{2+} and total Cr present in solution met the quality criteria of discharge of wastewater in natural waters, according to US-EPA (1984) and Portuguese law (Decreto-lei 236/98), after the fourth batch at pH 6.0.

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Chapter 8

Selective recovery of copper, nickel and zinc from ashes produced from *Saccharomyces cerevisiae* contaminated biomass used in the treatment of real electroplating effluents[7]

Abstract

The aim of this work was to seek an environmentally friendly process for recycling metals from biomass-sludges generated in the treatment of industrial wastewaters. This work proposes a hybrid process for selective recovery of copper, nickel and zinc from contaminated biomass of *Saccharomyces cerevisiae*, used in the bioremediation of electroplating effluents. The developed separation scheme comprised five consecutive steps: (1) incineration of the contaminated biomass; (2) microwave acid (HCl) digestion of the ashes; (3) recovery of copper from the acid solution by electrolysis at controlled potential; (4) recycle of nickel, as nickel hydroxide, by alcalinization of the previous solution at pH=14; (5) recovery of zinc, as zinc hydroxide, by adjusting the pH of the previous solution at 10. This integrated approach allowed recovering each metal with high yielder (> 99% for all metals) and purity (99.9, 92 and 99.4% for copper, nickel and zinc, respectively). The purity of the metals recovered allows selling them in the market or being recycled in the electroplating process without waste generation.

8.1. Introduction

Even though a big research effort has been promoted in the development of biological-based alternatives for the purification of metal-bearing effluents, a limited

[7] Submitted for publication (Journal of Hazardous Materials, reference: HAZMAT-D-10-01973, in revision)

attention has been dedicated to metals recovery for subsequent re-use or sale. Basically, two approaches can be pursued: (i) metals desorption from the biomass; (ii) biomass dissolution (with strong acids) or incineration. Metals desorption from the biomass allows achieving the regeneration of biomass and simultaneous metals recovery from the liquid phase. Theoretically, this process is desirable in order to keep the process costs down. In this sense, different desorption agents have been used, namely: diluted (0.1 M) mineral (HCl, HNO_3 and H_2SO_4) (Strandberg *et al.* 1981; Wilhelmi and Duncan 1995; Wilhelmi and Duncan 1996) and organic acids (CH_3COOH) (Ferraz *et al.* 2004), ethylenediaminetetraacetic acid (EDTA) (Strandberg *et al.* 1981; Ferraz *et al.* 2004) and carbonates (Strandberg *et al.* 1981; Wilhelmi and Duncan 1996). However, some practical difficulties (such as: damage of yeast cells as a result of the eluant treatment, successive reduction of yeast metal uptake during biosorption/desorption cycles and in some cases, like Cr^{3+}, lower capacity to concentrate the metals in a small volume of solution (Strandberg *et al.* 1981; Ferraz *et al.* 2004) have been found, which compromises the feasibility of the desorption process. On the other hand, when biomass is inexpensive, like the use of brewer's yeast cells (a by-product from a fermentative industry), its dissolution or incineration is advantageous because it generates a concentrated solution of heavy metals; this aspect is particularly important as it can determine the feasibility of metals recovery.

The combustion of microorganisms and subsequent recovery of metals from ashes, by acid leaching, seems to be a promissory approach. This process reduces the amount of residues and concentrates metals. Later on, metals can be recovered from acid solutions by separation processes such as electrolysis or chemical precipitation.

Few studies have been performed for recovering selectively more than two different metal ions (Tabak *et al.* 2003; Tokuda *et al.* 2008). Selective recovery of copper, nickel and zinc with sodium sulphides was investigated by Fukuta *et al.* (2004) [quoted by Tokuda *et al.* (2008)]; in this work, copper, nickel and zinc were

recovered with a selectivity of 94.5%, 65.9% and 75.9%, respectively. Further studies on the selective precipitation of copper, nickel and zinc, using sulphides, enhanced the selectivity of metal precipitation for values higher than 95% (Tokuda *et al.* 2008). However, the use of sulphides implies the application of adequate safety procedures since it is toxic and corrosive. In addition, the transport, storage and production of sulphides are expensive (Huisman *et al.* 2006).

The main aim of this work was to develop a nearly closed cycle for recycling selectively, with high yield and purity, heavy metals from yeast cells contaminated with copper, nickel and zinc, coming from the treatment of real electroplating effluents. For this purpose, contaminated biomass was, firstly, incinerated. Then, ashes were totally acid digested under microwave conditions. From this solution, copper, nickel and zinc were selective and efficiently recovered with high purity by implementing a hybrid separation process; this separation procedure combines electrodeposition followed by a sequential alkaline precipitation. Finally, the possibility of re-using the metals, with lower purity, in the electroplating process or selling them is discussed.

8.2. Materials and methods

8.2.1. *Incineration of contaminated biomass*

An effluent from an electroplating industry containing copper, nickel and zinc was treated with heat-inactivated brewing cells of *Saccharomyces cerevisiae*. After effluent treatment (as described in Chapter 6; effluent A), contaminated biomass was collected and dried at 45 ºC.

Kinetic incineration studies were performed at 550 ºC in a muffle furnace (Nabertherm, L5/C6), using incineration trays containing 15 g of dried contaminated biomass. At defined intervals of time, incineration trays were taken, cooled down in a

dessicator and weighed. This procedure was repeated until constant weight. These experiments were done in duplicate.

8.2.2. *Microwave digestion of ashes*

About 200 mg of ashes obtained as described above and 4.5 ml of HCl 1 M were placed in a polytetrafluoroethylene vessel. Digestion of ashes was performed in a microwave digestor (Anton Paar, Multiwave 3000), using one cycle of 20 minutes and another one of 45 minutes, both with continuous radiation of 800 W. Then, 4.5 ml of HCl 1 M was added and another cycle of digestion (45 minutes with a continuous radiation of 800 W) was applied. A new batch containing 200 mg of ashes was added to the previous acid solution and a new cycle of digestion was applied (45 minutes with a continuous radiation of 800 W). In all cases, the cycles were preceded by a heating ramp of 15 minutes (heating rate: 53 W/min). After the heating ramp, the final temperature ranged between 200 and 220 ºC.

To control the efficiency of the microwave acid digestion, copper, nickel and zinc concentrations were determined in the acid solution, after the digestion of the first and second batches of ashes, by AAS-FA, as previously described (Chapter 4, Section 4.2.4). The concentrations of copper, nickel and zinc obtained in the acid digested solution were 3.5, 2.8 and 4.3 g/l, respectively.

8.2.3. *Copper recovery by electrolysis at controlled potential*

Before copper electrolysis, the pH of the acid digested solution was raised up to pH 2. Copper electrolysis was conducted using a batch reactor containing three electrodes. The cathode was a platinum net; a platinum wire and an electrode of Ag/AgCl (KCl 3 M) were used as anode and reference electrodes, respectively.

Deposition of copper was attained by applying a constant cathodic potential of -0.6 V [Ag/AgCl (KCl 3 M] with a potentiostat (Autolab, PGSTAT 302). A volume of 40 ml of acid solution was used, under constant agitation (500 rpm), at 35 ºC or 40 ºC. Current intensity was monitorized along the deposition time; it was assumed that electrodeposition was finished when current stabilized. At the end of copper electrolysis, copper concentration was quantified in the remained acid solution by AAS-FA for determining the efficiency of metal recovery. For controlling the selective recovery of copper, nickel and zinc were quantified, by AAS-FA, after redissolving the deposited metal in nitric acid.

These experiments were done in duplicate.

8.2.4. *Computer chemical simulations*

All chemical speciation calculations were carried out as described in Chapter 4, Section 4.2.6. Computational simulations were performed considering the total concentrations of nickel and zinc, which remained in the acid solution after copper deposition, metal hydroxides complexes and the solubility product constants (Martell and Smith 2004).

8.2.5. *Selective recovery of nickel and zinc by alcalinization at pH 14*

After copper deposition, the mass of sodium hydroxide (NaOH) necessary to adjust the pH of the remaining solution up to 14 was theoretically calculated taken into account the amount of hydroxide necessary to raise the pH from 2 to 14 plus the amount of hydroxide necessary to convert nickel and zinc to nickel hydroxide [$Ni(OH)_2$ (s)] and tetrahydroxozincate(II) [$Zn(OH)_4^{2-}$ (aq.)], respectively; due to the high ionic strength of the solution, calculation of pH was performed considering the

OH^- activity instead of OH^- concentration. The pH of the solution was adjusted by addition of small pellets of solid NaOH in order to minimize volume variation. Then, the suspension was centrifuged (2800 *g*, 10 minutes) and nickel recovered as a precipitate of $Ni(OH)_2$. Nickel precipitate was washed two times with 10 ml of NaOH, at pH=14, and different times (one to four) with 10 ml of NaOH at pH 10; after each washing step, the solid was recovered by centrifugation (2800 *g,* 10 minutes). Subsequently, the pH of the supernatant solution was adjusted to 10, by addition of HCl 37%, in order to precipitate zinc, as zinc hydroxide, $Zn(OH)_2(s)$. After that, the suspension was centrifuged (2800 *g*, 10 minutes) to recover the precipitate of zinc hydroxide. This precipitate was further washed different times (one to three) with NaOH, at pH 10, and recovered by centrifugation (2800 g, 10 minutes).

To determine the efficiency of nickel and zinc recovery, as well as to characterize the purity of the precipitates, concentrations of copper, nickel and zinc were determined by AAS-FA, after appropriate dissolution of the precipitates in HNO_3 20% (v/v). Sodium and chlorides can also be present as impurities in the precipitates. Sodium concentration was determined by Emission Atomic Spectroscopy (EAS) in a Perkin Elmer AAnalyst 400 spectrometer (Norwalk, CT, USA). Concentration of chlorides was determined as the difference between the total amount of the dried precipitate weighted gravimetrically and the sum of copper, nickel, zinc (all in the form of metal hydroxide) and sodium quantified in the precipitate. All determinations were done at least in duplicate.

8.3. Results and discussion

Effluent A, containing 20, 22 and 25 mg/l of copper, nickel and zinc, respectively, was efficiently treated, as described in Chapter 6. This process allowed reducing metal concentrations, in the treated effluent, to values below the Portuguese

and US-EPA legal limits of discharge (Chapter 6). However, these heavy metals were only transferred from a liquid (effluent) to a solid phase (yeast cells) generating toxic sludges with high metal concentrations. Thus, a process to recover efficient and selectively copper, nickel and zinc from the contaminated biomass was required to be developed.

For heavy metals recovery, the first step should be the extraction of metals from biomass. As it was reported above, desorption of metals from biomass presents some difficulties. Alternatively, biomass can be dissolved using concentrated mineral acids or incinerated. In the present work, biomass incineration and subsequent digestion of ashes was chosen due to the following reasons: (i) the incineration originates a drastic reduction of the mass of the residues generated as a consequence of the bioremediation process used on the treatment of the effluent; (ii) the subsequent acid digestion of the ashes leads to the achievement of a concentrated solution of heavy metals, which is a key factor on the feasibility of metals recovery process.

8.3.1. *Incineration of contaminated biomass*

Contaminated yeast cells were incinerated and converted into ashes at 550 ºC to assure that organic matter was eliminated without formation of extremely recalcitrants compounds (Paul *et al.* 2006). Preliminary incineration studies with different amounts of contaminated biomass (2, 10 and 15 g) have shown similar periods of time to complete incineration of biomass. Thus, further incineration studies were performed using 15 g of contaminated biomass.

Kinetics studies of mass thermal decreasing were performed at 550 ºC. As it can be seen in Figure 8.1, after 15 minutes and 4 hours of incineration, a mass reduction of 83.1 and 97.6% was achieved, respectively. Total mineralization of the

organic matter (incineration until a constant weight was attained) present in the sludges was obtained after 12.5 days (300 hours) of incineration, which corresponded to a final mass decrease of 98.7%. The increasing of the incineration time up 300 hours did not reveal a great decrease of the biomass (Δ weight = 1.1%) when compared with the decrease observed after 4 hours. From these results, we concluded that an incineration time of 4 hours should be used because it allows a significant mass decrease and saving of energy consumption.

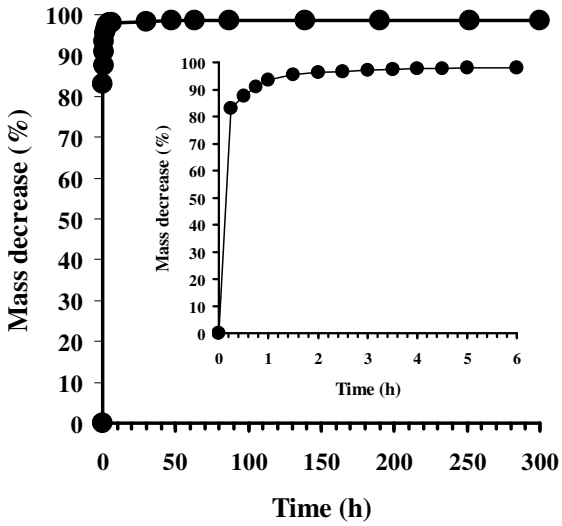

Figure 8.1. Evolution of yeast cells mass loss. 15 g of *S. cerevisiae* brewer's yeast cells, used in the bioremediation of an electroplating effluent and loaded with heavy metals, was incinerated at 550 ºC until constant weight. Each point represents the mean of two replicates.

8.3.2. *Ashes digestion*

A microwave acid digestion with closed vessels was used to maximize the leaching of copper, nickel and zinc from the ashes. Closed vessels allow high pressures, which results in temperatures higher than the acid boiling point; as a consequence, the digestion process is accelerated (Luque-García and Luque de

Castro 2003). This method also consumes a small volume of acid, which assures a relatively low sample dilution. Consequently, high metals concentrated solutions are obtained.

Because sulphuric, nitric and hydrochloric acids are economic and widely used in the electroplating industries, we started by evaluating the feasibility of using each one of these acids in the ashes digestion. Copper, nickel and zinc react with diluted sulphuric acid to form metal sulphates, which crystallise at room temperature (Brown 1974). So, sulphuric acid could not be used in the acid digestion of the ashes because it would not allow a subsequent selective recovery of each metal. Additionally, nitric acid did not meet the requirements for being used in the subsequent selective recovery of metals by electrolysis; in the presence of nitrates, besides copper, nitrates reduction also occurs in the cathode according to reactions [8-1] and [8-2], respectively (Chang 1994):

$$Cu^{2+}_{(aq)} + 2e^- \leftrightarrows Cu_{(s)} \qquad E^0 = 0.34\ V \qquad [8\text{-}1]$$

$$NO_3^-{}_{(aq)} + 4H^+_{(aq)} + 3e^- \leftrightarrows NO_{(g)} + 2H_2O_{(l)} \qquad E^0 = 0.96\ V \qquad [8\text{-}2]$$

Nitric oxide, $NO_{(g)}$, rapidly reacts with atmosferic oxygen and is converted to brown nitrogen dioxide, $NO_{2(g)}$, a toxic gas by inhalation. Moreover, gas evolution at the cathode decreases the electroplating rate and can create porous metal deposits (Tan 1993). Hydrochloric acid can be used to digest the ashes without formation of crystals. Additionally, subsequent recovery of copper can be obtained by electrolysis without chlorides gas evolution at the anode because chlorides only evolutes at a more anodic potential than water oxidation:

$$Cl_{2(g)} + 2e^- \leftrightarrows 2Cl^-_{(aq)} \qquad E^0 = 1.36\ V \qquad [8\text{-}3]$$

$$O_{2(g)} + 4H^+_{(aq)} + 4e^- \leftrightarrows 2H_2O_{(l)} \qquad E^0 = 1.23\ V \qquad [8\text{-}4]$$

Previous calculations have shown that HCl 1 M should be enough to dissolve totally the heavy metals present in the ashes. Previous studies allowed optimizing other experimental conditions (volume of acid, time of digestion and power conditions) (data not shown). The digestion of 400 mg (200 plus 200 mg) of ashes, with HCl 1 M under the experimental conditions described in Section 8.2.2, allowed the total dissolution of copper, nickel and zinc. The acid digestion of the ashes resulted in a solution containing 3.5, 2.8 and 4.3 g/l of copper, nickel and zinc, respectively.

In the next steps, selective recovery of copper, nickel and zinc from the acid digestion solution, followed by the evaluation of possible metals reinsertion in the electroplating production process, was studied.

8.3.3. *Selective recovery of copper, nickel and zinc*

8.3.3.1. Copper recovery by electrolysis at a controlled potential

Electrolysis can be applied as a metal recovery process when sufficiently large differences in the metals reduction potentials of the metals to be removed occurs (Suzuki *et al.* 1995). Preliminary studies have shown that copper deposition already occurs at a cathodic potential of -0.4 V/[Ag/AgCl (KCl 3 M)] at room temperature. However, an increase of the cathodic deposition potential improves copper deposition rate (Doulakas *et al.* 2000) but can eventually co-deposite nickel and zinc. It was verified that no co-deposition of nickel and zinc occurs at a cathodic potential of -0.6 V/[Ag/AgCl (KCl 3 M]; thus, this potential was selected for recovering copper from the acid digested solution. For this purpose, firstly, the pH of the acid digested solution was raised up to pH 2. Subsequently, copper was electrodeposited at 35 ºC or 40 ºC and constant agitation (500 rpm).

Figure 8.2. shows the variation of the current intensity with time. In the first 60 minutes of deposition, no appreciable decrease of the current intensity was observed for both temperatures assayed. This fact can be explained by the high concentration of Cu^{2+} in solution that must undergo previous reduction to Cu^{+} before being reduced to Cu^{0} and deposited in the platinum electrode (Doulakas *et al.* 2000). Then, a rapid decrease of the current intensity was observed for both temperatures tested. The kinetics of copper deposition at 40ºC was faster than at 35 ºC (Figure 8.2).

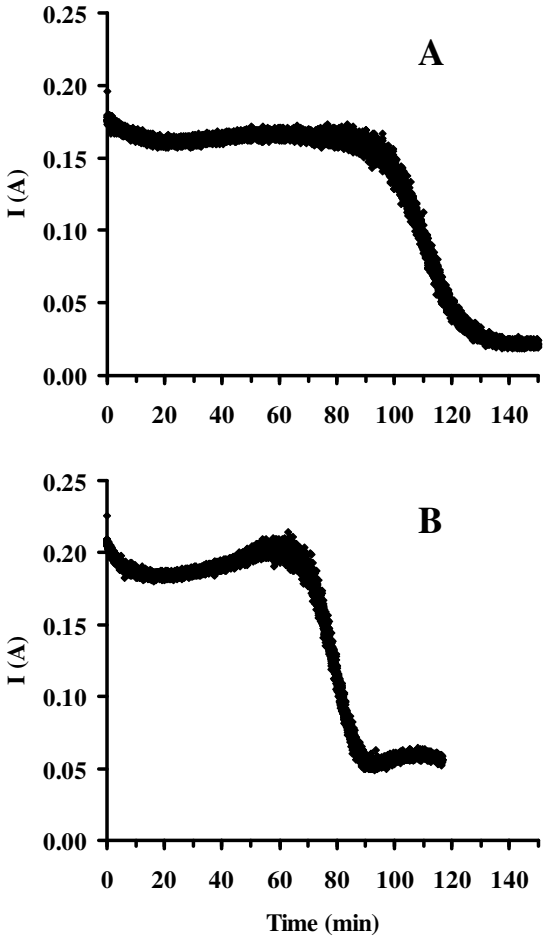

Figure 8.2. Evolution of intensity current profile of copper electrodeposition. The pH of the acid digestion solution was adjusted at 2 and copper was electrodeposited at -0.6 V/[Ag/AgCl (KCl 3M)], at 35 ºC (**A**) or 40 ºC (**B**).

When the current intensity stabilized, it was assumed that copper electrodeposition was finished. In fact, after 90 minutes of electrolysis, at 40 ºC, a residual copper concentration of 30 mg/l was present in solution, which corresponded to a recovery of 99.1%. At 35 ºC, the kinetic of copper deposition was slower and after 130 minutes of electrolysis, a copper residual concentration of 7 mg/l was achieved, corresponding to 99.8% of metal removal. These data demonstrate that the rise of cell temperature from 35 to 40 ºC, reduced considerably the time of electrolysis (about half-hour) without a significant decrease of the yield of copper recovery.

The purity of the metallic copper, recovered at 40 ºC, was very high, 99.9%, containing minor amounts [0.05 and 0.07% (w/w)] of nickel and zinc, respectively. Similar copper purity (higher than 99.5 mol%) had been reported by Doulakas *et al.* (2000) when copper was recovered, under potentiostatic conditions, from a synthetic chloride solution containing a mixture of cadmium, copper, lead and zinc ions.

The results obtained in the present work show that copper can be efficient and rapidly recovered, as metallic copper with high purity, from the acid digestion solution containing multiple metal ions (copper, nickel and zinc) using a cathodic potential of -0.6V [Ag/AgCl (KCl 3 M)] under mild temperature (40 ºC) and agitation (500 rpm) conditions.

The obtained metallic copper can be sold in the market or reintroduced in the copper plating baths (sulfate copper acid baths, fluoborate acid baths, copper pyrophosphate-plating baths and brass plating solutions) at the production process (Homer 1994).

If electroplating industries use baths of nickel-zinc alloys, the acid digested solution remained after the electrodeposition of copper, which contains nickel and zinc, can be directly reintroduced in the production process to prepare those plating

baths. If this application is not practicable, selective recovery of nickel and zinc is required.

8.3.3.2. Selective recovery of nickel and zinc

Firstly, the selective recovery of nickel from the remaining acid digested solution by electrolysis, at a controlled potential, was evaluated. The feasibility of the process would allow recovering nickel, in the metallic form, while zinc would remain in solution. The gap of potential between the standard reduction potentials of nickel and zinc (reactions [8-5] and [8-6], respectively, presented below) is 0.50 V:

$$Ni^{2+}_{(aq)} + 2e^- \leftrightarrows Ni_{(s)} \qquad E^0 = -0.25\ V \qquad [8\text{-}5]$$

$$Zn^{2+}_{(aq)} + 2e^- \leftrightarrows Zn_{(s)} \qquad E^0 = -0.76\ V \qquad [8\text{-}6]$$

This means that, from a theoretical point of view, deposition of nickel without simultaneous codeposition of zinc should be possible. In order to determine the cathodic deposition potential where nickel and zinc are reduced, polarization studies were carried out applying a linear potential sweep from 0 to -2 V *versus* Ag/AgCl (KCl 3 M) using single metal ion component solutions at pH 2. Nickel started to deposit at a cathodic potential of -0.95 V/[Ag/AgCl (KCl 3 M)] and zinc at a cathodic potential of -1.2 V/[Ag/AgCl (KCl 3 M)]. These experimental results evidenced that the gap of potential between the beginning of nickel and zinc deposition is very tight (0.25 V) and does not allow a complete separation of these two metals by electrolysis.

As it was already mentioned above, selective sequential recovery of nickel and zinc processes were recently developed and described in the literature to separate these metal ions, as metal sulfides precipitates from multi-metal systems (Tokuda *et*

al. 2008). However, the handling of sulphides poses severe adequate safety procedures and its transport, storage and production are expensive (Huisman *et al.* 2006).

Taking into account the impossibility of nickel and zinc recovery by electrolysis, with high purity, it was decided to develop a separation process based on an alkaline precipitation scheme. For this purpose, theoretical chemical distributions diagrams were done, using the MINEQL+ program, assuming the concentrations of nickel and zinc present in the remaining acid digested solution, 2.8 and 4.3 g/l respectively, in the pH range between 5 and 16 (Figure 8.3). Chemical distributions diagrams predict that both metal ions start to precipitate at about pH 7. For pH value higher than 13, zinc hydroxide starts to redissolve, as complexes of $Zn(OH)_4^{2-}(aq)$, being totally soluble at about pH=14 (Figure 8.3-B). At this pH, nickel should still be found precipitated as $Ni(OH)_2(s)$ (Figure 8.3-A). The analysis of these chemical distribution diagrams pointed out that a new strategy for selective recovery of nickel and zinc could be drawn based on the different solubility behaviour of nickel and zinc under alkaline conditions.

According to the information obtained from the chemical distributions diagrams of nickel and zinc (Figure 8.3), the pH of the remaining acid digested solution was raised up to pH=14. Since the final pH was very high and thus could not be measured accurately, the mass of sodium hydroxide necessary to adjust the pH was theoretically calculated (for further details see Section 8.2.5).

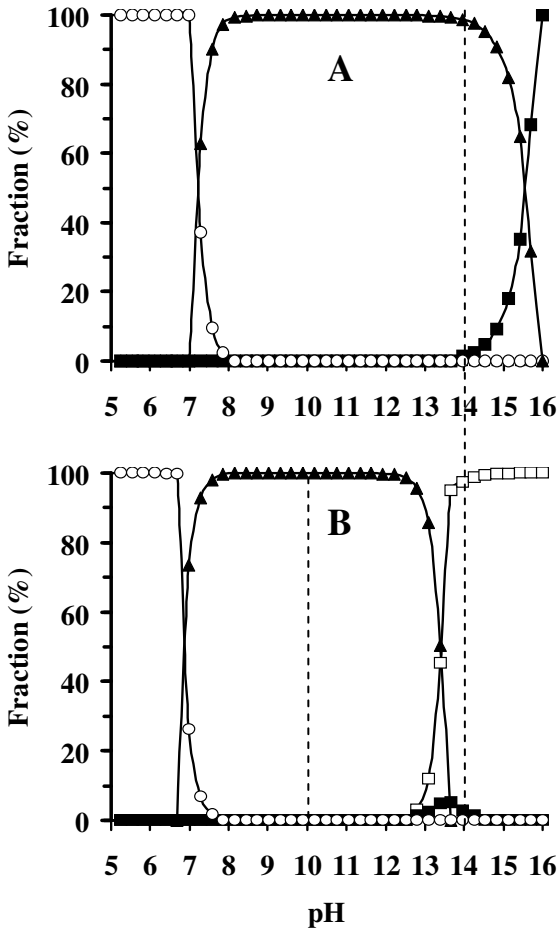

Figure 8.3. Species distribution diagrams of nickel and zinc at different pH values. 2.8 g/l of nickel (**A**) and 4.3 g/l of zinc (**B**) were considered. The different species of metals in solution were calculated with a chemical equilibrium computer program (MINEQL+) and assuming the total concentrations of metals present in the acid digested solution: free metal ion, $M^{2+}(aq)$ (○), $M(OH)_{2(s)}$ (▲) $M(OH)_3^{-}{}_{(aq)}$ (■) and $M(OH)_4^{2-}{}_{(aq)}$ (□).

Under these conditions, 97% of nickel was recovered (Table 8.1), as nickel hydroxide, and zinc remained totally soluble. Nickel hydroxide was recovered with a purity of 8% (Table 8.1); sodium and chlorides constituted the main impurities (about 90%) present in the precipitate (Figure 8.4).

Table 8.1. Recovery and purity of the precipitate of nickel hydroxide.

	Without wash	Washing steps at pH 10*			
		1	2	3	4
Recovery (%)	97 ± 5	100 ± 1	96 ± 5	98 ± 6	100 ± 1
Purity (%)	8 ± 1	40 ± 1	63 ± 7	77 ± 2	92 ± 7

* The precipitate was previously washed two times with NaOH at pH 14; subsequent washing steps were carried out at pH 10.

Minor amounts (less than 2%) of copper and zinc were present in the precipitate (Figure 8.4).

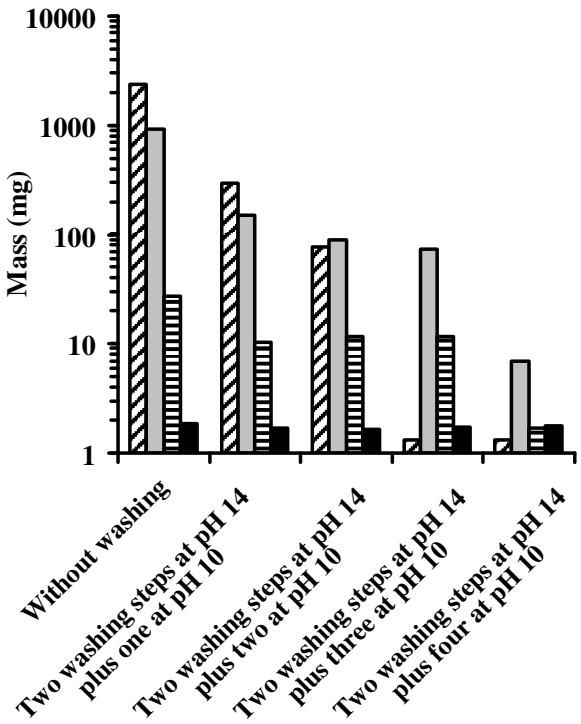

Figure 8.4. Evolution of the decrease of contaminants present in the nickel hydroxide precipitate with the washing steps. Amounts of chlorides (bar with sloping lines), sodium (grey bar), zinc hydroxide (bar with horizontal lines) and copper hydroxide (black bar), present in the nickel hydroxide precipitate.

At this stage, this precipitate can be reused for adjusting the nickel concentration in the Watts electroplating baths; these baths usually present a

concentration of 30-60 g/l of $NiCl_2.6H_2O$ and sodium does not affect the electrodeposition performance (Homer 1994). Additional washing steps for recovering nickel hydroxide are necessary to be implemented if a more pure compound is desirable.

In order to eliminate possible vestigial amounts of zinc impurities, the precipitate of nickel hydroxide was firstly washed with a sodium hydroxide solution at pH=14. Since at this pH, zinc is totally soluble (Figure 8.3-B), this washing approach allows eliminating any possible amount of zinc, which remained in the precipitate. Next, the precipitate was washed with a sodium hydroxide solution at pH 10 for eliminating sodium and chlorides from the precipitate. As it can be seen in Table 8.1**,** the purity of the precipitate increased significantly (from 8 to 92%) with no appreciable reduction of the recovery yield. In addition, Figure 8.4 also evidences that the increase of purity was mainly due to the leaching of sodium and chlorides from the precipitate. The nickel hydroxide obtained in this work, with a purity of 92%, can be sold for industrial purposes (*e.g.* production of nickel-cadmium and nickel-manganese batteries) (U.S-D.H.H.S 2005).

Chemical distribution diagram of zinc (Figure 8.3-B) evidences, that at pH 10, zinc should be completely precipitated as $Zn(OH)_2(s)$. So, in the next step, the pH of the remained supernatant solution was adjusted at pH 10 and zinc was totally recovered as a precipitate of zinc hydroxide (Table 8.2).

Table 8.2. Recovery and purity of the precipitate of zinc hydroxide.

	Washing steps at pH 10			
	0	**1**	**2**	**3**
Recovery (%)	100 ± 3	95 ± 6	88 ± 4	100 ± 6
Purity (%)	55.3 ± 0.1	97 ± 2	99 ± 1	99.4 ± 0.8

The precipitate was recovered with a purity of 55.3%, being sodium and chlorides the main impurities present in the precipitate (Figure 8.5); only vestigial amounts of copper and nickel were present in the precipitate (Figure 8.5). The recovered zinc hydroxide can be reintroduced in the chloride electroplating baths, since these baths present a concentration of 120 g/l of NaCl (Homer 1994).

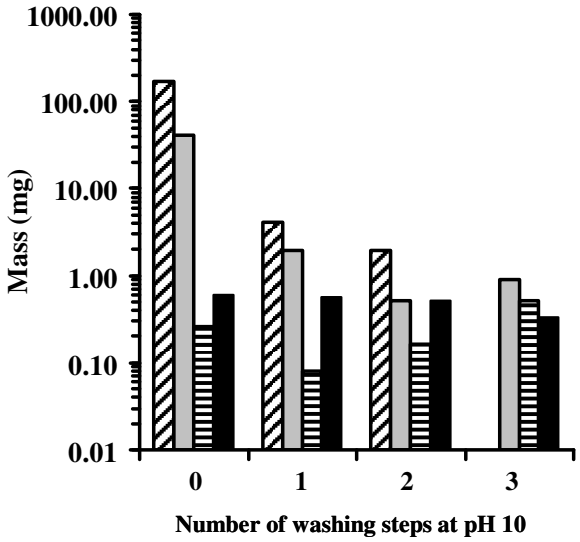

Figure 8.5. Evolution of the decrease of contaminants present in the zinc hydroxide precipitate along the washing steps at pH 10. Amounts of chlorides (bar with sloping lines), sodium (grey bar), nickel hydroxide (bar with horizontal lines) and copper hydroxide (black bar), present in the zinc hydroxide precipitate.

If more pure zinc hydroxide is desirable, additional washing steps are needed. For this purpose, the precipitate was washed with a sodium hydroxide solution at pH 10 using one, two and three washing steps (Table 8.2 and Figure 8.5). After one washing step, the purity of the precipitate increased to 97% (Table 8.2); this increase of purity was mainly due to the leaching of sodium and chlorides from the precipitate (Figure 8.5). When two or three washing steps were implemented, almost all the remained sodium and chlorides present in the precipitate were leached and the purity of the precipitate increased to 99 and 99.4%, respectively, with no appreciable reduction of the recovery yield (Table 8.2).

The introduction of washing steps for purifying nickel and zinc hydroxides generate small volumes of alkaline rinsing waters containing concentrations of nickel and zinc above the limits of discharge (Tables 8.3 and 8.4, respectively). In order that no residues are generated during the recycling process, we propose the incorporation of these rinsing waters plus the aqueous NaCl at pH= 10, which remained after zinc hydroxide recovery, together with the effluents to be treated by biosorption.

Table 8.3. Concentration of metals determined in the washing waters of nickel hydroxide.

Metal concentration (mg/l)	**Washing waters***					
	1st	**2nd**	**3rd**	**4th**	**5th**	**6th**
Cu	1.5	0.41	0.35	0.13	0.04	0.08
Ni	0.7	1.1	1.2	1.2	4.9	4.8
Zn	1.5×10^3	5.9×10^2	3.0×10^2	15	1.4	0.4

* The first two washing steps were performed with NaOH at pH 14; the other ones were done with NaOH at pH 10.

Table 8.4. Concentration of metals determined in the washing waters of zinc hydroxide.

Metal concentration (mg/l)	**Washing waters**		
	1st	**2nd**	**3rd**
Cu	0.1	0.2	0.1
Ni	0.1	0.2	0.6
Zn	7.8	7.3	10

The washing steps were performed with NaOH at pH 10.

8.3.4. *Environmental friendly selective recovery of copper, nickel and zinc*

Based on the above studies, an environmental friendly process for selective recovery of copper, nickel and zinc from yeast cells, used in the bioremediation of electroplating effluents, was developed. A schematic diagram of the process is shown in Figure 8.6. The major steps of this process are: (1) incineration of contaminated

biomass; (2) acid digestion of the ashes; (3) recovery of copper from the acid solution by electrolysis at a controlled potential; (4) recovery of nickel by alcalinization of the solution at pH 14; (5) zinc recovery by pH adjustment at 10.

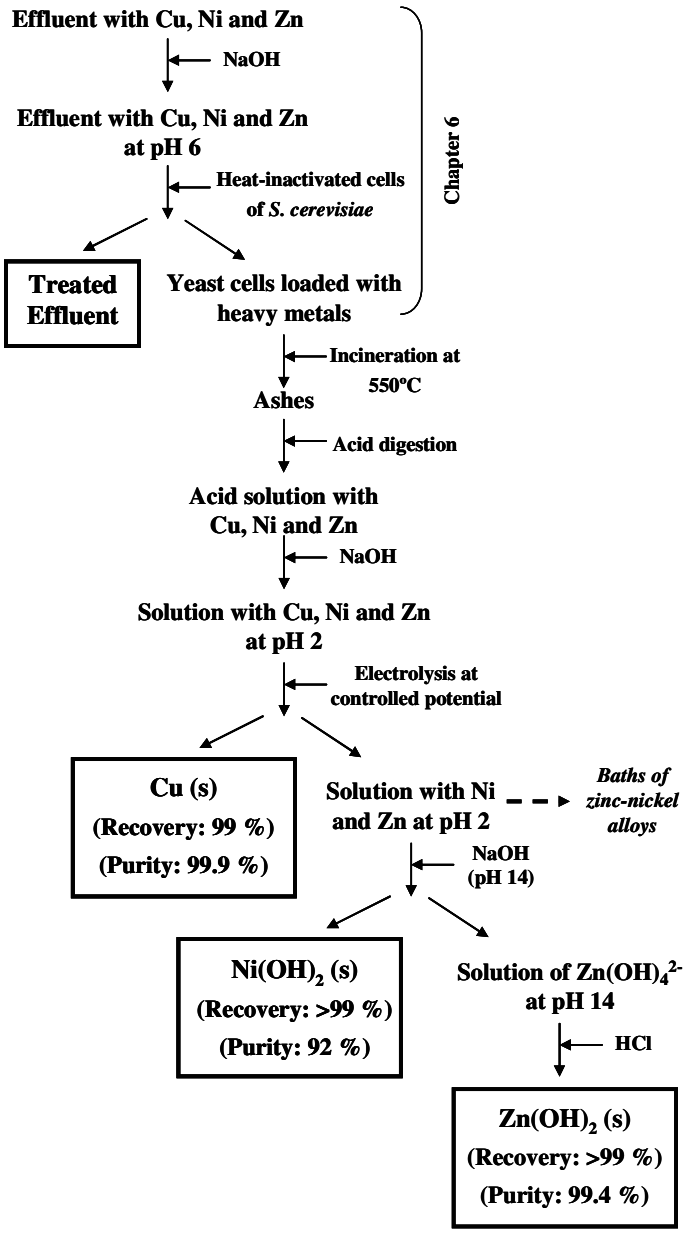

Figure 8.6. Diagrammatic representation of the overall clean procedure proposed in the present work for removing and recovering selectively copper, nickel and zinc from contaminated yeast cells produced in the bioremediation of electroplating effluents.

This process allowed an efficient and selective recovery of the different metals from contaminated biomass. Copper, nickel and zinc can be re-sailed as pure copper or nickel and zinc hydroxides, respectively; alternatively, metals can be send back and reused in the electroplating process. In both cases, the selective recovery proposed is not only economically profitable but also environmental beneficial.

8.4. Conclusions

In the present work, a nearly closed cycle for recovering selectively each metal was completed. For this purpose, contaminated yeast cells were incinerated, the ashes were acid digested in a microwave assisted process and the metals present in the concentrated solution were sequential and selectively recovered by electrolysis (metallic copper) and alkaline precipitation (nickel and zinc as metal hydroxides).

The incineration step allowed, after 4 hours, a reduction of 98% of the toxic mass. Additionally, metals (copper, nickel and zinc) present in the solution obtained after microwave acid digestion of the ashes were concentrated about 170 times.

This closed cycle constitutes an effluent-free process, which allows selective recovery of copper, nickel and zinc with high yield (>99% for all metals) and purity (99.9, 92 and 99.4% for copper, nickel and zinc, respectively). The developed process can be easily implemented and constitutes a positive approach to the wastewater treatment since it combines the minimization of environmental liabilities with financial benefits (recovered metals can be re-sailed or re-used).

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Chapter 9

Selective recovery of chromium, copper, nickel and zinc from an acid solution using an environmentally-friendly process [8]

Abstract

Real electroplating effluents contain multiple metals. An important point related with the feasibility of the bioremediation process is linked with the strategy to recovery selectively metals. In this work, a multi-metal solution, obtained after microwave acid digestion of the ashes resulted from the incineration of *Saccharomyces cerevisiae* contaminated biomass, was used to recovery selectively chromium, copper, nickel and zinc. The acid solution contained 3.8, 0.4, 2.8 and 0.2 g/l of Cr^{3+}, Cu, Ni and Zn, respectively. Copper was recovered (97.6%), as metallic copper, by electrolysing the solution at a controlled potential. Subsequently, 87.9% of nickel was recycled, as a precipitate of nickel hydroxide, by simultaneous alcalinization of the solution at pH 14 and oxidation of Cr^{3+} to Cr^{6+}. 82.7 % of the total amount of zinc was recovered, as a precipitate of zinc hydroxide, and 95.4% of Cr^{6+} remained in solution, as chromate, after adjusting the pH of the remaining solution at pH 10. This process allowed a selective and efficient recovery of chromium, copper, nickel and zinc from an acid solution using combined electrochemical and chemical processes. Financial advantages can be achieved since the metals recovered can be sailed or reintroduced in the electroplating baths.

[8] Submitted for publication

9.1. Introduction

Cells of *Saccharomyces cerevisiae* seems to be a promissory type of biomass for the bioremediation of wastewaters containing heavy metals, as they have ability to remove a wide variety of metals from synthetic (Zouboulis *et al.* 2001; Chen and Wang 2008; Machado *et al.* 2008; Ruta *et al.* 2010) and real effluents (Stoll and Duncan 1997; Parvathi and Nagendran 2007; Machado *et al.* 2010b). It was shown that flocculent brewing strains of *S. cerevisiae* seems particularly appropriated as they have a higher metal uptake than non-flocculent strains (Soares *et al.* 2002). On the other hand, heat-killed biomass (at 45 ºC) of brewing yeast cells present higher metals removal than live cells (Machado *et al.* 2009) and retain their flocculation properties, which overcomes the problem of cell separation process after effluent treatment (Machado *et al.* 2008). Recently, it was demonstrated that flocculent brewing yeast cells are able to treat efficiently complex industrial effluents loaded with several heavy metals (Machado *et al.* 2010a; Machado *et al.* 2010b).

After heavy metals have been removed by yeast cells from industrial effluents, heavy metals recovery from biomass allows metals recycling and contributes to reduce the costs since the metals can be reintroduced in the industrial process or re-sailed. The efficiency of metals recovery is largely dependent of the concentration of metals present in the solution after being extracted from the biomass. Desorption of heavy metals from yeast cells have been attempted by several authors using different eluants such as mineral and organic acids or complexing agents (Strandberg *et al.* 1981; Wilhelmi and Duncan 1996; Ferraz *et al.* 2004), as it was already mentioned in Chapter 8; however, the lower capacity to concentrate metals strongly limits the feasibility of the recovery process. Alternatively, the amount of yeast biomass loaded with heavy metals (coming from the bioremediation process) can be strongly reduced by incineration; then, the acid digestion of the ashes generates a concentrated acid metals solution.

Usually industrial effluents contain multiple heavy metals. An important point related to metals recovery is linked with the strategy to remove selectively metals from the solution. The simultaneous recovery of several heavy metals is indubitably a more ambitious and difficult challenge. Thus, it is not surprising that a limited work has been devoted to this objective. The selective recovery of copper, nickel and zinc by sequential precipitation using sulphides was described (Tokuda *et al.* 2008). However, sulphides toxicity and consequently the safety aspects related with its handling strongly impairs its practical application (Huisman *et al.* 2006).

In the present work, a multistage process, which combines electrochemical and chemical methods, was developed for recovering selective and efficiently chromium, copper, nickel and zinc from an acid solution. The acid solution resulted from the incineration of contaminated brewing cells of *Saccharomyces cerevisiae* loaded with heavy metals.

9.2. Materials and methods

9.2.1. *Incineration of yeast cells and acid digestion of ashes*

Brewing cells of *Saccharomyces cerevisiae,* used in the bioremediation of an electroplating effluent (Chapter 6, effluent B), were dried at 45 ºC and then incinerated at 550 ºC, in a muffle furnace (Nabertherm, L5/C6), until constant weight. Ashes were digested in a microwave digester as described in Chapter 8, Section 8.2.2.

After the digestion of the ashes, total chromium, copper, nickel and zinc concentrations were determined in the acid solution by AAS-FA, as described in Chapter 4, Section 4.2.2, in order to control the efficiency of the digestion. The

obtained acid solution contained 3.8, 0.4, 2.8 and 0.2 g/l of Cr^{3+}, Cu, Ni and Zn, respectively.

This experiment was performed in duplicate.

9.2.2. *Copper recovery by electrolysis*

Copper was recovered by electrolysis, at 40ºC, under potentiostatic conditions, as described in Chapter 8, Section 8.2.3.

In order to determine the efficiency of metal recovery, copper concentration was quantified in the remained acid solution by AAS-FA. For controlling the selective recovery of copper, total chromium, nickel and zinc were quantified in the metallic copper, by AAS-FA, after redissolving it in nitric acid.

All experiments and determinations were done at least in duplicate.

9.2.3. *Computer chemical simulations*

All chemical speciation calculations were carried out in a similar way, as described in Chapter 4, Section 4.2.6. Computational simulations were performed considering the total heavy metals (chromium, nickel and zinc quantified in the remained acid digested solution after copper deposition) concentrations, metal hydroxides complexes and the solubility product constants (Martell and Smith 2004).

9.2.4. *Nickel recovery by simultaneous alcalinization at pH 14 and oxidation of Cr^{3+} to Cr^{6+}*

Since the final pH was very high (pH 14), the mass of sodium hydroxide necessary to adjust the pH was theoretically calculated taken into account the amount of hydroxide necessary to raise the pH from 2 to 14 plus the amount of hydroxide necessary to convert chromium, nickel and zinc to chromate [$CrO_4^{2-}{}_{(aq)}$], $Ni(OH)_{2(s)}$ and $Zn(OH)_4^{2-}{}_{(aq)}$, respectively; due to the high ionic strength of the solution, calculation of pH was performed considering the OH^- activity instead of OH^- concentration. Then, small pellets of solid sodium hydroxide were added. After all sodium hydroxide was dissolved, an excess of hydrogen peroxide [5 times more the amount of Cr^{3+}] was added. Finally, the suspension was boiled during 15 minutes, in order to decompose the excess of hydrogen peroxide, and centrifuged at 2800 *g* for 10 minutes, for recovering the precipitate of nickel hydroxide, $Ni(OH)_{2(s)}$. Nickel hydroxide was firstly washed two times with 10 ml of NaOH at pH 14, followed by four washing steps with 10 ml of NaOH at pH 10; the precipitate was recovered by centrifugation at 2800 *g* during 10 minutes.

Oxidation of Cr^{3+} to Cr^{6+} was controlled by determining the amount of total chromium and Cr^{6+} in the remained supernatant solution. Total Cr and Cr^{6+} were quantified, as described in Chapter 4, Section 4.2.4.

For characterization of nickel hydroxide and determination of nickel recovery, concentrations of total chromium, copper, nickel, sodium and zinc were measured in solution after appropriate dissolution in HNO_3 20% (v/v) and dilution of a dried (at 105 ºC until constant weight) weighted amount of nickel hydroxide. Sodium concentration was determined by EAS and total chromium, copper, nickel and zinc were measured by AAS-FA, as described in Chapter 8, Section 8.2.5. The amount of chlorides present in the precipitate was calculated as the difference between the total amount of a dried (at 105 ºC until constant weight) precipitate weighted

gravimetrically and the sum of copper, nickel, zinc (all in the form of metal hydroxide) and sodium quantified in the precipitate.

All experiments and determinations were done at least in duplicate.

9.2.5. *Zinc and chromium recovery by adjustment of pH at 10*

After nickel recovery, the remained supernatant solution, containing Cr^{6+} and Zn, was adjusted at pH 10 by addition of HCl 37% followed by centrifugation at 2800 *g* for 10 minutes. Zinc was recovered as a precipitate of zinc hydroxide, $Zn(OH)_{2(s)}$, and chromium remained in solution as $CrO_4^{2-}{}_{(aq)}$.

The amount of zinc recovered was determined by AAS-FA, after appropriate dissolution of part of the dried (at 105 ºC until constant weight) precipitate in HNO_3 20% (*v*/*v*) and dilution.

For characterization of chromate solution and quantification of chromium recovery, total chromium, copper, nickel, sodium and zinc were determined in the remained supernatant solution as it was described above. Chlorides were analysed according to the Standard Methods (APHA *et al.* 1998) by the argentometric method. All experiments and determinations were done at least in duplicate.

9.3. Results and discussion

In this work, an acid solution, obtained after microwave acid (hydrochloric acid was used) digestion of the ashes obtained from the incineration of contaminated biomass used to treat effluent B (Chapter 6), was used. The acid solution contained 3.8, 0.4, 2.8 and 0.2 g/l of Cr^{3+}, Cu, Ni and Zn, respectively. This solution was used

to develop a nearly closed cycle for recovering selectively chromium, copper, nickel and zinc (Figure 9.1).

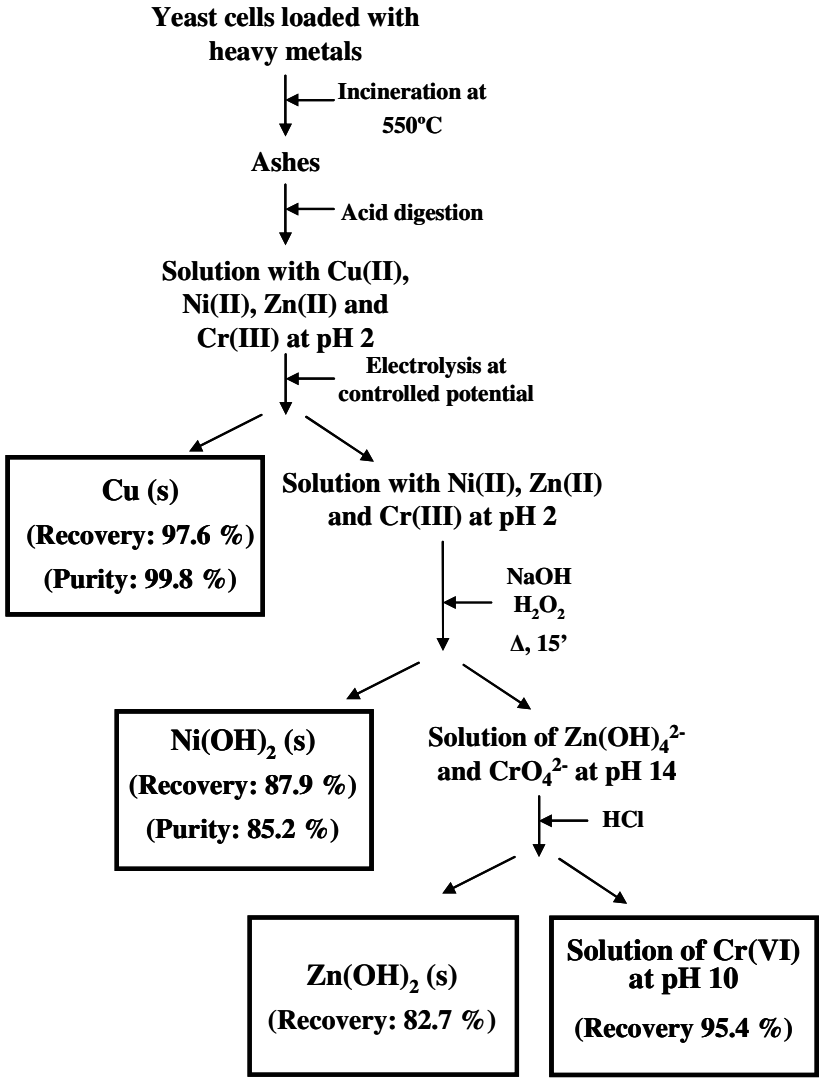

Figure 9.1. Diagrammatic representation of the overall environmentally friendly process proposed in the present work. The process allows an efficient and selective recovery of chromium, copper, nickel and zinc from an acid solution.

In a first attempt, copper was electrolysed from the multi-metal acid solution after previous pH adjustment of the acid digested solution at pH 2. After one hour of electrolysis, a residual concentration of copper, 9 mg/l, remained in the acid

solution. This corresponded to a recovery of 97.6% of copper (Figure 9.1), which is in agreement with the results from other authors.

Thus, Suzuki *et al.* (1995) reported the complete removal of copper from a mixture of copper, nickel and zinc by electrolysis at a controlled potential; in the same way, Aydin *et al.* (1998) reported the electrodeposition of 99.3% of copper from a solution containing bismuth, cadmium, cobalt, copper, nickel and zinc.

In the present work, the purity of the metal recovered was 99.8% (Figure 9.1), containing minor amounts of chromium, nickel and zinc (Table 9.1). Doulakas et al. (2000) had reported a similar copper purity (higher than 99.5%) when copper was recovered from a multi-element solution containing cadmium, copper, lead and zinc ions, at a controlled potential.

Table 9.1. Main impurities presented in metallic copper, nickel hydroxide and chromate solution.

Metals	Contaminants (%, w/w)					
	Na	**Cl**	**Cu**	**Ni**	**Zn**	**Cr**
Cu(s)	**–**	**–**	**–**	**0.050**	**0.060**	**0.070**
$Ni(OH)_2$ (s)	**0.30**	**11.4**	**0.40**	**–**	**0.40**	**2.3**
CrO_4^{2-} (aq)	**5.78**	**18.2**	**$\leq 3.0x10^{-5}$**	**$\leq 3.0x10^{-5}$**	**$\leq 4.0x10^{-5}$**	**–**

The high purity of the final product obtained allows its introduction in the market. On the other hand, it can also be introduced in several types of copper plating baths (namely in sulfate copper acid baths, fluoborate acid baths as well as in copper pyrophosphate-plating baths and brass plating solutions) (Homer 1994).

Then, the possibility of recovering selectively chromium, nickel and zinc by chemical precipitation was evaluated. For this purpose, chemical speciation distribution diagrams, using the MINEQL+ program, were drawn assuming the

concentrations of chromium, nickel and zinc present in the remained acid solution (Figure 9.2). The analysis of figure pointed out that at pH 6, Cr^{3+} should be totally precipitated, as $Cr(OH)_{(3)}$ (Figure 9.2-C), whereas nickel (Figure 9.2-A) and zinc (Figure 9.2-B) should remain soluble, as free metal ions, in solution. From a theoretical point of view, these results suggested that Cr^{3+} could be selectively recovered by adjusting the pH of the solution obtained after electrolysis at pH 6; nickel and zinc ions should remain in solution. However, experimental studies evidenced that part of nickel and zinc coprecipitated with chromium hydroxide (Annex C-1), which unabled this strategy. On the other hand, the analysis of Figures 9.2-A to 9.2-C evidenced that whatever the pH at which the solution was adjusted, at least two metals would be remained in the same phase (precipitated or soluble).

From these results, we definitively concluded that a new strategy should be developed to recovery selectively the three metals. The analysis of chemical speciation distribution diagram of Cr^{6+} (Figure 9.2-D) together with the chemical speciation distribution diagrams for nickel (Figure 9.2-A) and zinc (Figure 9.2-B) pointed out that nickel could be selectively recovered, as a precipitate of nickel hydroxide, from the remaining solution [chromium and zinc will remain totally soluble as anions of $CrO_4^{2-}{}_{(aq)}$ and $Zn(OH)_4^{2-}{}_{(aq)}$ at about pH 14. It is described in the literature (Vogel 1987) that oxidation of Cr^{3+} to Cr^{6+} can be easily promoted using an excess of hydrogen peroxide, under alkaline conditions, according to the equation below:

$$2\,[Cr(OH)_4]^-_{(aq)} + 3H_2O_{2(aq)} + 2OH^-_{(aq)} \rightarrow 2CrO_4^{2-}{}_{(aq)} + 8H_2O_{(l)} \qquad [9\text{-}1]$$

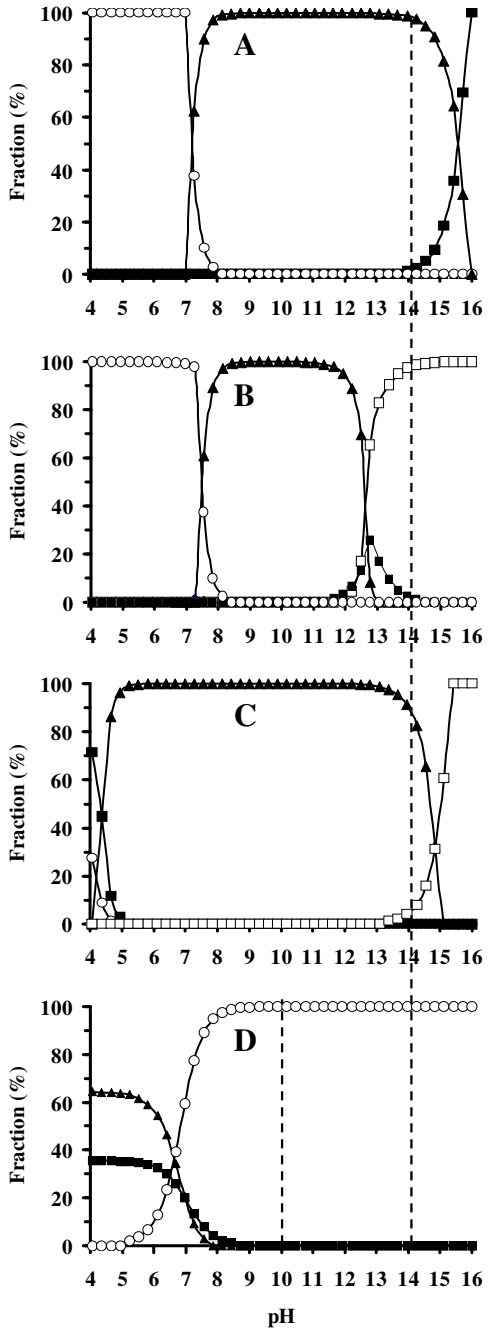

Figure 9.2. Species distribution diagram of 2.8 g/l of Ni (A), 0.2 g/l of Zn (B) and 3.8 g/l of Cr^{3+} (C) or Cr^{6+} (D), at different pH values. The different species of metals in solution were calculated with a chemical equilibrium computer program (MINEQL+) and assuming the total concentrations of metals remained in the acid digested solution after copper electrolysis. Ni or Zn species: free metal ion, $M^{2+}_{(aq)}$ (○), $M(OH)_{2(s)}$ (▲), $M(OH)_3^-{}_{(aq)}$ (■) and $M(OH)_4^{2-}{}_{(aq)}$ (□). Cr^{3+} species: $Cr^{3+}_{(aq)}$ (○), $Cr(OH)^{2+}_{(aq)}$ (■), $Cr(OH)_{3(s)}$ (▲) and $Cr(OH)_4^-{}_{(aq)}$ (□). Cr^{6+} species: $CrO_4^{2-}{}_{(aq)}$ (○), $Cr_2O_7^{2-}{}_{(aq)}$ (▲) and $HCrO_4^-{}_{(aq)}$ (■).

All these facts allowed us to design another strategy to recovery selectively chromium and zinc. This strategy was based on the oxidation of Cr^{3+} to Cr^{6+} under stronger alkaline conditions.

Due to this, we decided to increase the pH of the suspension up to 14 and add an excess of peroxide hydrogen (Figure 9.1). Under these conditions, 95.4% of Cr^{3+} was oxidized to Cr^{6+} and 87.9% of Ni was recovered, as nickel hydroxide, with a purity of 85.2 % (Figure 9.1). Chromium and chlorides were the main contaminants present in the nickel hydroxide (Table 9.1). Minor amounts of copper, sodium and zinc were also detected. The nickel hydroxide, obtained under the present conditions, can be reused to adjust nickel concentration in the Watts baths, which present a concentration of 30-60 g/l of $NiCl_2.6H_2O$ (Homer 1994).

After Ni recovery, the remained solution was mainly constituted by high amounts of Cr^{6+} and small amounts of zinc. Chemical speciation distribution diagrams of Zn (Figure 9.2-B) and Cr^{6+} (Figure 9.2-D) predicted that, at pH 10, Zn should be totally precipitated, as $Zn(OH)_{2\,(s)}$, and Cr^{6+} will remain in the supernatant, as $CrO_4^{2-}{}_{(aq)}$. So, the pH of the remained solution was adjusted to pH 10 with concentrated hydrochloric acid. After this procedure, 82.7% of the zinc initially present in the solution was recovered (Figure 9.1). Since the initial solution only contained 0.2 g/l of zinc, the final amount of zinc hydroxide recovered was very low**.** So, no attempts to purify this amount of zinc hydroxide were performed; in this case, the recovered zinc hydroxide should be reintroduced in a subsequent initial acid digested solution. On the other hand, if significant amounts of zinc are present in the initial acid solution, large amounts of zinc hydroxide will be recovered. In this case, if a high purified final product is required, the recovered zinc hydroxide can be washed using NaOH at pH 10 in a similar way as it was described in Chapter 8.

The concentration of chromium present in the recovered cromate solution was 2.6 g/l**,** which corresponded to a recovery of 95.4% (Figure 9.1). This solution contained chlorides and sodium ions, as major contaminants, and vestigial amounts

of Cu, Ni and Zn (Table 9.1). This solution can be reused in the preparation of chromium plating solution of conventional (150-400 g/l of CrO_3) and cocatalyzed baths (150-400 g/l of CrO_3) (Homer 1994).

9.4. Conclusions

In this work, a multi-step process for recovering selectively chromium, copper, nickel and zinc from an acid solution was developed. For this purpose, a solution obtained after microwave acid (hydrochloric acid was used) digestion of the ashes produced from the incineration of contaminated biomass used to treat effluent B (Chapter 6), was used. The acid solution contained 3.8, 0.4, 2.8 and 0.2 g/l of Cr^{3+}, Cu, Ni and Zn, respectively.

The developed process comprised three steps: (1) selective recovery of copper, as metallic copper, by electrolysis at controlled potential; (2) selective recovery of nickel, as nickel hydroxide, by simultaneous alcalinization at pH 14 and oxidation of Cr^{3+} to $Cr^{6+}$of the previous solution; (3) selective recovery of zinc, as zinc hydroxide, and of Cr^{6+}, as a solution of cromate, by adjusting the pH of the previous solution at pH 10. With this process, final recoveries of 95.4, 97.6, 87.9 and 82.7% of Cr^{6+}, Cu, Ni and Zn, respectively, were obtained. The purity of the metals recovered allows reintroducing them in the electroplating process.

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Chapter 10

Main conclusions and future work

10.1. Conclusions

The cells of *Saccharomyces cerevisiae* NCYC 1364 retain their flocculation ability in the presence of different heavy metals. So, yeast flocculation can be used as a fast, easy and economic technology of cell separation in industrial effluents containing Cu, Ni, Zn, Cd and Cr facilitating recovery and recycling of biomass. This natural aggregation can be applied as a separation process for both live and dead cells. Furthermore, it is possible to use different configurations of biomass reactors without the risk of washout and to profit equipment that already exists in industries to treat effluents such as sedimentation tanks.

A killing temperature of 45 ºC was the most appropriated to inactivate cells of *Saccharomyces cerevisiae* NCYC 1364 since no loss of sedimentation characteristics occurred and morphologic and cytological changes were minimized (Chapter 2).

Kinetic and equilibrium studies evidenced that dead yeast cells (at 45 ºC) presented higher metals uptake than live cells. The increase of metal uptake capacity can be explained by the loss of membrane integrity that exposes other metal-binding sites present inside the cells. Kinetics studies have also shown that for live and dead cells a contact time of 30 minutes is enough to achieve equilibrium between metals and biomass, for all metal studied, except for live cells with Cu^{2+}, where the equilibrium was attained after 60 minutes (Chapter 3). Thus, dead biomass was chosen for being applied on effluents bioremediation since it combines an efficient removal with a rapid separation biomass from reaction mixture after treatment.

Having as objective to optimize effluents treatment, the influence of inorganic ligands present in real electroplating effluents on the removal of chromium, copper, nickel and zinc by yeast biomass was predicted by metal chemical speciation studies. High concentrations of carbonates, chlorides, fluorides, and nitrates in the effluents do not compete with biomass for copper, nickel and zinc. However, the presence of phosphates or fluorides can reduce the efficiency of chromium biosorption. Sulphates, at molar concentrations higher than that of biomass, can compete with biomass for nickel (Chapter 4). The presence of fluoride anions in electroplating effluents may also decrease the efficiency, or even preclude, of bioremediation of Cr^{3+} by yeast cells. Nevertheless, the presence of fluorides does not affect the bioremediation of effluents containing Cu^{2+} and Ni^{2+} by *Saccharomyces cerevisiae* heat-inactivated yeast cells (Chapter 5). Metal chemical speciation also allowed designing best experimental conditions for removing efficiently the metals with high efficiency, namely biomass concentration as well as the number of batches necessary to remove efficiently metals to the limits of discharge of USA and Portuguese laws (Chapters 4, 6 and 7).

A hybrid technology, which combines chemical precipitation with subsequent biosorption by dead biomass at 45º C, at pH 6.0, was developed for removing heavy metals uni-element (Ni) or multi-elements (Cr, Cu, Ni and Zn) from real electroplating effluents (Chapter 6). For effluents containing Cr and/or Cu, chemical precipitation followed by two biosorption batch steps will be enough to reduce metal(s) concentration(s) to values below the legal limits of discharge, whatever the concentration of Cr and/or Cu present in the original effluent.

After treatment of effluents containing (i) only Ni; (ii) Cu, Ni and Zn; (iii) Cu, Ni, Zn and trivalent Cr; (iv) Cu, Ni, Zn and hexavalent and trivalent Cr, the quality criteria of discharge of wastewater in natural waters, according US-EPA and Portuguese law was met in less than four batches.

For effluents containing Cr^{3+} and Cr^{6+} (among other metals), two strategies were developed: (i) reduction of Cr^{6+} to Cr^{3+}, pH adjustment to 6.0 and subsequent treatment with a serial batch of biomass (Chapter 6) or (ii) without a pre-treatment process of Cr^{6+} reducing (Chapter 7). In the last case, Cr^{6+} was selectively removed by yeast biomass at pH~2; subsequently, pH of the effluent was raised up to 6.0 and treated with a serial batch of biomass for removing the other metals: Cu^{2+}, Cr^{3+} and Ni^{2+} (Chapter 7). The process (i) is desirable for effluents containing Ni, since a higher efficiency of metals removal was obtained with a lower number of batches of biomass. In the case of wastewaters containing Cr^{6+} and Cr^{3+}, but without Ni, selectively removal of Cr^{6+} at pH~2, followed by removal of other metals at pH 6.0 is an attractive process since it avoids the Cr^{6+} reduction step, with the consequent minimization of the costs.

For recovering selective and sequentially each metal from biomass an environmentally-friendly method was developed. Yeast biomass loaded with metals was incinerated at 550 ºC for 4 hours and the ashes were acid digested in a microwave assisted process. The metals present in the concentrated solution were sequential and selectively recovered by electrolysis (metallic copper) and alkaline fractional precipitation (nickel and zinc as metal hydroxides). If trivalent chromium is present in the effluent, simultaneous alcalinization at pH 14 and oxidation of Cr^{3+} to Cr^{6+}aprovides selective recovery of chromium as a solution of chromate.

In conclusion, a nearly closed cycle for removing and recovering selectively copper, nickel, zinc and chromium from electroplating wastewaters was developed. This closed cycle constitutes an effluent-free process, which allows selective recovery of chromium, copper, nickel and zinc with high yield and purity. The purity of the metals recovered allows selling them in the market or being recycled in the electroplating process without waste generation. The developed process can be easily implemented and constitutes a good alternative to the wastewater treatment since it

combines the minimization of environmental responsibilities with financial benefits (recovered metals can be re-sailed or re-used).

10.2. Future work

Having as final objective applying this technology on the removal and recovery of heavy metals from real effluents at electroplating industries, it is necessary to undertake scale-up studies. Before transferring the procedures developed from laboratory to production scale, an intermediate scale, pilot scale, should be used to optimize process conditions and adequate industrial equipments.

Meanwhile, laboratorial studies with others metals can be performed having as objective to extend the applicability of this process to other industrial effluents metal-loaded.

Annexes

Annex A

Annex A-1: Settling profile of live cells of *Saccharomyces cerevisiae* NCYC 1364 in presence of different metals

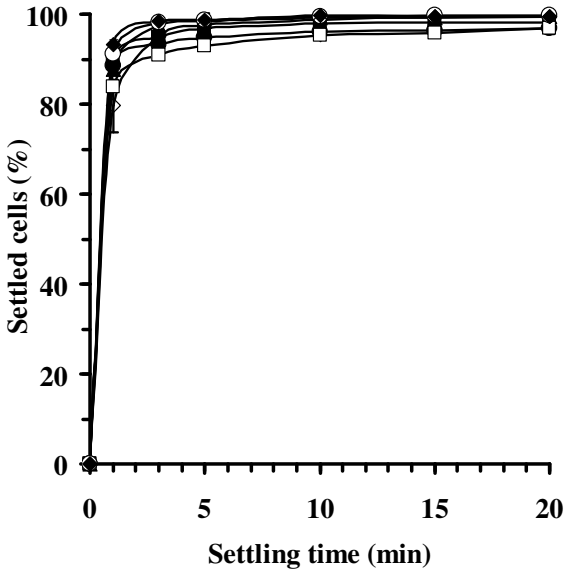

Figure A-1. Time course of sedimentation live cells of *Saccharomyces cerevisiae* NCYC 1364 in the presence of different cations. Yeast biomass (5 g dry weight/l) was suspended in MES pH buffer (10 mM, pH 6.0), containing 0.2 mM of each cation: (■) Ni^{2+} + Zn^{2+}; (●) Ni^{2+} + Cd^{2+}; (▲) Ni^{2+} + Cr^{3+}; (□) Zn^{2+} + Cd^{2+}; (○) Cd^{2+} + Cr^{3+}, (◊) Zn^{2+} + Cr^{3+}, (♦) Ca^{2+} (control ion). Values are the mean of three replicates of two independent experiments; standard deviations are presented (n =6).

Annex A-2: Settling profile of live cells of *Saccharomyces cerevisiae* NCYC 1364 in presence of different effluents

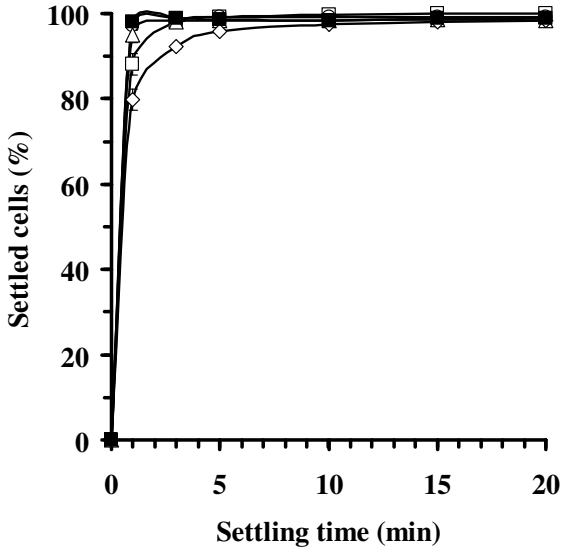

Figure A-2. Time course of sedimentation of live cells of *Saccharomyces cerevisiae* NCYC 1364 in synthetic and real effluents adjusted at pH 6.0. Yeast biomass (5 g dry weight/l) was suspended in: (□) Synthetic effluent (Chapter 2, Section 2.3.1); (○) Electroplating effluent (Chapter 4, Section 4.3.1); (Δ) Electroplating effluent A (Chapter 6, Section 6.3.1) (◊) Electroplating effluent B (Chapter 6, Section 6.3.1) and (■) and MES pH buffer (10 mM, pH 6.0) with 4 mM of Ca^{2+}(control). Values are the mean of three replicates of two independent experiments; standard deviations are presented (n =6).

Annex A-3: Settling profile of heat treated cells at 45 ºC of *Saccharomyces cerevisiae* NCYC 1364 in presence of different effluents

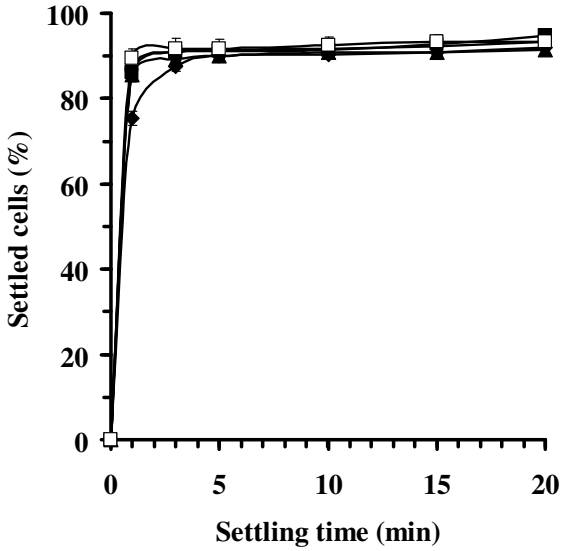

Figure A-3. Time course of sedimentation of dead cells (killed at 45 ºC)) of *Saccharomyces cerevisiae* NCYC 1364 in synthetic and real effluents adjusted at pH 6.0. Yeast biomass (5 g dry weight/l) was suspended in: (■) Synthetic effluent (Chapter 2, Section 2.3.1); (●) Electroplating effluent (Chapter 4, Section 4.3.1); (▲) Electroplating effluent A (Chapter 6, Section 6.3.1); (♦) Electroplating effluent B (Chapter 6, Section 6.3.1) and (□) MES pH buffer (10 mM, pH 6.0 with 4 mM of Ca^{2+}(control). Values are the mean of three replicates of two independent experiments; standard deviations are presented (n =6).

Annex B

Annex B-1: *Saccharomyces cerevisiae* viability during copper accumulation

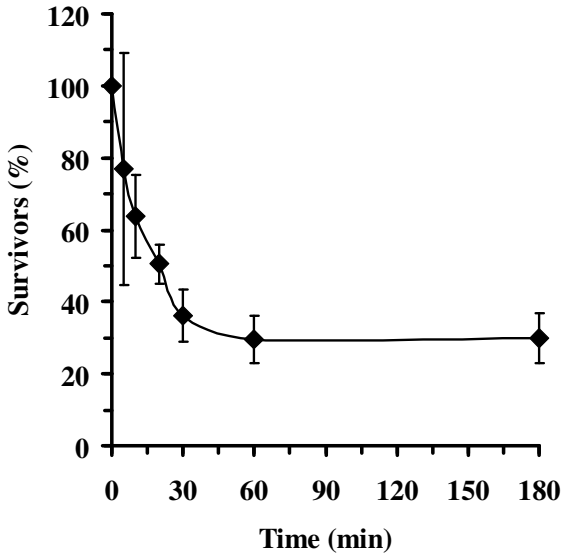

Figure B-1. Viability of *Saccharomyces cerevisiae* NCYC 1364 during copper accumulation (5 mg/l). The viability of the cells was assessed by the classical spread-plating method. Each point represents the mean of two experiments performed in duplicate; standard deviations are presented (n=4).

Annex C

Annex C-1: Chromium recovery by adjusting pH at 6

Table C-1. Chromium recovery, as chromium hydroxyde [$Cr(OH)_3(s)$], by adjusting pH at 6. Nickel and zinc coprecipitated with chromium at pH 6 inviabilizing an effective recovery.

Metal	% of metal precipitated
Cr	37.2
Ni	65.8
Zn	99.0