Ana Filipa Benedito da Assunção

BIOLOGICAL AND CHEMICAL STRATEGIES FOR THE RECOVERY OF PRECIOUS METALS AS NANOPARTICLES

UNIVERSIDADE DO ALGARVE

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“Biological and Chemical strategies for the recovery of precious metals as nanoparticles”

Declaração de Autoria do Trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Ana Filipa Benedito da Assunção

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FCT
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and to my grandparents.

"Sem sonhos, a vida não tem brilho.
Sem metas, os sonhos não têm alicerces.
Sem prioridades, os sonhos não se tornam reais."
(Augusto Cury)

LIST OF PUBLICATIONS

This thesis is based on the following original articles:

Maria Clara Costa, **Ana Assunção**, Ana M. Rosa da Costa, Carlos Nogueira and Ana Paula Paiva (2013) Liquid-Liquid Extraction of Platinum from Chloride Media by N,N′-Dimethyl-N,N′-Dicyclohexyltetradecylmalonamide. *Solvent Extraction and Ion Exchange*, 31(1): 12-23.
DOI: 10.1080/07366299.2012.700588

Maria Clara Costa, Ruben Almeida, **Ana Assunção**, Ana Maria R da Costa, Carlos Nogueira and Ana P Paiva (2016) N,N´-tetrasubstituted succinamides as new molecules for liquid-liquid extraction of Pt(IV) from chloride media. *Separation and Purification Technology*, 158: 409–416.
DOI: 10.1016/j.seppur.2015.12.035

Mónica Martins†, **Ana Assunção**†, Hélio Martins, António Pedro Matos and Maria Clara Costa (2013) Palladium recovery as nanoparticles by an anaerobic bacterial community. *Journal of Chemical Technology and Biotechnology*, 88(11): 2039–2045. (†Mónica Martins and Ana Assunção contributed equally to this study)
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Ana Assunção, Maria Clara Costa and Jorge Carlier (2015) Application of urea-agarose gel electrophoresis to select non-redundant 16S rRNAs for taxonomic studies: palladium (II) removal bacteria. *Applied Microbiology and Biotechnology*, published online 21 November 2015
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Ana Assunção, Ana Matos, Ana M. Rosa da Costa, António Candeias and Maria Clara Costa. A bridge between liquid-liquid extraction and the use of bacterial communities for palladium and platinum recovery as nanosized metal sulphides. *Hydrometallurgy*. (*under review*) - (CHAPTER 6)

Ana Assunção, Bernardete Vieira, João P Lourenço and Maria Clara Costa. Recovery of gold (0) nanoparticles from aqueous solutions using effluents from a bioremediation process. (*submitted*)* – (CHAPTER 7)
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Ana Assunção, Bernardete Vieira, Gonçalo Vitor, João P. Lourenço, Olinda C. Monteiro and Maria Clara Costa. Green synthesis and characterization of Ag_2S nanoparticles and Ag_2S/TiO_2 nanocomposites aiming radiation based applications. (*in preparation*) - (CHAPTER 8)

Abstract

The present work explored the potential of chemical (solvent extraction) and biological (using anaerobic bacterial community) technologies, aiming the removal/recovery of precious metals from aqueous solutions.

Solvent extraction or liquid-liquid extraction was used to find a suitable platinum and palladium extractant, to be applied to the treatment of aqueous solutions containing these metals. Thus, N,N'-dimethyl-N,N'-dicyclohexyltetradecylmalonamide (DMDCHTDMA), N,N'-dicyclohexyl-N,N'-dimethylsuccinamide (DMDCHSA) and N,N'-dimethyl-N,N'-diphenylsuccinamide (DMDPHSA) were tested and proved to be efficient at extracting platinum (IV) from HCl solutions. Platinum (IV) was then successfully stripped from DMDCHTDMA using 1 M HCl solution, and from DMDCHSA and DMDPHSA, using seawater.

These extractants proved to be less efficient for palladium (II) removal, thus, for this metal, biological strategies were tested. A palladium (II)-resistant bacterial community, able to biorecover palladium from solutions, was found. The phylogenetic analysis showed that this community was mainly composed by bacteria close to *Clostridium* species, however, bacteria affiliated to genera *Bacteroides* and *Citrobacter* were also identified.

The potentialities of combining solvent extraction and the use of bacterial communities for palladium and platinum recovery were also demonstrated. Pd(II) and Pt(IV) in aqueous phases were efficiently extracted to organic phases. The metals were then stripped with seawater, precipitated and recovered using a filtered solution from bacterial growth. The successful precipitation of palladium (II) and platinum (IV), as nanosized PdS and PtS_2 particles, was accompanied by sulphide concentration decrease in the bacterial growth solution.

Biological processes, using effluents from an acid mine drainage bioremediation treatment system, were tested for gold recovery from aqueous solutions. The use of these effluents, with dissolved biogenic sulphide, proved to be an excellent alternative for Au(III) recovery as Au(0) nanoparticles (NPs).

Finally, biogenic sulphide, from the effluents tested before, was also used to successfully synthesize Ag_2S NPs and Ag_2S/TiO_2 nanocomposite. The Ag_2S NPs and Ag_2S/TiO_2 nanocomposite obtained have semiconductor properties and can theoretically be used for radiation based applications.

Keywords: Precious Metals, Platinum Group Metals, Solvent Extraction, Bioremoval/biorecovery, Nanoparticles

Resumo

O principal objetivo do presente trabalho foi explorar as potencialidades de tecnologias química e biológica, para a remoção/recuperação de metais preciosos de soluções aquosas. A abordagem química focou-se no uso da técnica de extração líquido-líquido, também conhecida como extração por solventes. Assim, foram estudados alguns extratantes, diamidas, de forma a extrair metais do grupo da platina, no caso, platina e paládio, a partir de soluções de ácido clorídrico. A utilização de consórcios de bactérias anaeróbias para a remoção e/ou recuperação de metais preciosos (ouro, prata, paládio e platina) a partir de soluções aquosas, preferencialmente sob a forma de NPs, também foi explorada. Para além destas duas abordagens, ainda foi testada a combinação de ambas as metodologias, química e biológica, na recuperação de metais do grupo da platina a partir de soluções aquosas.

Os metais preciosos (ouro, prata e metais do grupo da platina – PGM - platina, paládio, ruténio, irídio e ródio) têm vindo a desempenhar um papel de destaque na sociedade atual, dadas as suas inúmeras aplicações, nomeadamente como catalisadores em processos orgânicos, componentes de valor acrescentado em ligas metálicas, conversores catalíticos em veículos, nas indústrias química, farmacêutica, petrolífera e eletrónica, assim como em joalharia. Esta crescente procura dos metais preciosos, conduziu ao aumento da escassez das suas fontes primárias. Por conseguinte, e uma vez que o valor económico destes metais assim o justifica, a investigação de processos para a sua separação tem vindo a crescer, com particular incidência na utilização de materiais ou métodos inovadores, que permitam a sua recuperação seletiva e rentável. A implementação de processos que permitam a valorização de efluentes, ou a reciclagem de diferentes materiais no período final da sua vida útil, que contenham metais preciosos é extremamente urgente e importante.

Diversas abordagens têm vindo a ser estudados para a remoção e recuperação de metais preciosos, no entanto, métodos químicos como a extração por solventes e a troca iónica, e métodos biológicos usando bactérias anaeróbias, têm ganho especial foco.

Alguns autores têm investigado o uso de derivados de malonamidas N,N’-tetrasubstituídas na extração de iões metálicos presentes em soluções de lixiviação cloretadas, usando a extração por solventes.

No presente trabalho, foi seguida a abordagem da extracção líquido-líquido, em busca de extratantes para a platina e para o paládio, que possam ser aplicados a soluções de lixiviação, nomeadamente resultantes do tratamento de fontes secundárias. Para tal, um derivado de malonamida N,N'-tetrassubstituído - N,N'-dimetil-N,N'-diciclohexiltetradecilmalonamida (DMDCHTDMA) – e dois derivados de succinamidas N,N´-tetrasubstituídas - N,N'-diciclohexil-N,N'-dimetilsuccinamida (DMDCHSA) e N,N'-dimetil-N,N'-difenilsuccinamida (DMDPHSA) – foram sintetizados e o seu potencial extractante foi investigado na extração líquido-líquido de Pt(IV), a partir de soluções cloretadas. Os resultados revelaram que a platina (IV) pode ser extraída de forma eficiente por qualquer um dos três extractantes testados. Posteriormente, a platina foi re-extraída, com sucesso, da solução da DMDCHTDMA carregada com o metal, através de um simples contacto com uma solução de HCl 1M. A platina também foi re-extraída num só passo e com sucesso das fases orgânicas carregadas de DMDCHSA e DMDPHSA, usando água do mar.

Estes extractantes, apesar de serem bastante eficientes na extração da platina, não se mostraram tão eficientes na extração de paládio, pelo que foram testados métodos biológicos para a remoção de paládio (II) de soluções aquosas.

Diversos métodos biológicos têm vindo a ser propostos e estudados, apresentando algumas vantagens relativamente aos métodos químicos, nomeadamente serem mais seguros a nível ambiental (produzem menos resíduos ou efluentes) e serem uma tecnologia de baixo custo (normalmente operam nas condições ambientais, sem que seja necessário gastos energéticos adicionais e sem necessidade de reagentes). Assim, a biorecuperação baseada na utilização de microrganismos tem sido considerada como alternativa potencialmente viável. De facto, é conhecido que os microrganismos podem ser utilizados na remoção de iões metálicos de soluções aquosas através de reações de precipitação ou de redução, que conduzem à formação de NPs metálicas.

Tendo como base as considerações anteriores, no presente trabalho foram realizados estudos com vista à remoção e recuperação de metais preciosos a partir de soluções aquosas, usando consórcios de bactérias anaeróbias. Assim, lamas provenientes de Estações de Tratamento de Águas Residuais foram enriquecidas, de modo a promover preferencialmente o crescimento de bactérias redutoras de sulfato, bem conhecidas como tendo capacidade bioremediadora de metais. A comunidade enriquecida foi exposta a diferentes concentrações de paládio (II), tendo sido possível encontrar uma comunidade bacteriana não só

resistente ao paládio (~20 mg/L), como também com capacidade para o remover da solução. A análise filogenética permitiu concluir que este consórcio bacteriano é essencialmente composto por bactérias da espécie *Clostridium*, não obstante, bactérias do género *Bacteroides* e *Citrobacter* também foram identificadas.

Posteriormente, testou-se pela primeira vez as potencialidades da combinação da extração líquido-líquido com o uso de comunidades bacterianas para a recuperação de paládio e platina, sob a forma de NPs de sulfuretos metálicos. Estes sulfuretos metálicos de dimensões nanométricas têm aplicações funcionais já conhecidas e demonstradas. Assim, o Pd(II) e a Pt(IV) presentes em soluções aquosas foram extraídos eficientemente para fases orgânicas compostas por DMDCHSA e por DMDCHTDMA em 1,2-dicloroetano, respetivamente. Os metais, agora presentes nas fases orgânicas, foram re-extraídos, com elevado sucesso, utilizando água do mar. Para a recuperação do metal da solução aquosa utilizou-se uma solução filtrada, proveniente do crescimento e atividade do consórcio bacteriano obtido anteriormente. A precipitação, praticamente total, dos metais, foi acompanhada por uma diminuição do sulfureto presente na solução (proveniente do crescimento bacteriano), ocorrendo assim a formação de NPs de PdS e de PtS_2.

Para a recuperação do ouro de soluções aquosas utilizou-se um processo baseado na utilização do efluente proveniente de um processo de bioremediação de uma Água Ácida de Mina (AMD). A utilização deste efluente, contendo sulfureto gerado biologicamente, num processo de recuperação de ouro, provou ser uma excelente alternativa para a obtenção de ouro metálico sob a forma de NPs. Deste modo, este método permite simultaneamente tratar um efluente e obter um produto de elevado valor económico, como são as NPs de Au(0).

Por fim, este mesmo efluente foi utilizado de forma similar à utilizada na recuperação de NPs de Au(0), para a bioremoção de prata de meios aquosos, tendo originado síntese de NPs de Ag_2S. Uma vez que esta síntese apresentou inicialmente, num sistema em *batch*, um rendimento de ~100%, foi realizado um *scale-up* para um sistema acoplado a jusante do processo de bioremediação de AMD em contínuo (anteriormente já utlizado na recuperação do ouro). Esta síntese permitiu obter NPs de Ag_2S ainda mais pequenas (tamanhos entre 15-24 nm) do que as obtidas em *batch* (tamanhos até 54 nm). Também foi realizada com sucesso a síntese de nanocompósitos de Ag_2S/TiO_2, acoplada ao sistema de bioremediação de AMD. As partículas do nanocompósito Ag_2S/TiO_2

apresentaram dimensões compreendidas entre 30 e 60 nm, justificadas pelas partcículas de TiO_2 apresentarem na sua superfície partículas de Ag_2S, como mostrado pelos resultados da microscopia electrónica. Todas as NPs sintetizadas provaram apresentar características semicondutoras, o que lhes confere a possibilidade teórica de serem utilizadas em processos fotocatalíticos. Com este trabalho, foi possível encontrar bons extratantes e agentes de re-extração, nunca antes reportados, para a extração de platina a partir de soluções cloretadas. Também demonstrou-se ser possível obter consórcios bacterianos, a partir de amostras ambientais, resistentes e com capacidade de remover paládio de soluções aquosas. Concluiu-se também que a combinação da extracção líquido-líquido e do uso de comunidades bacterianas permite recuperar a platina e o paládio presentes em soluções aquosas, sob a forma de NPs de sulfuretos metálicos. Por fim, provou-se que um efluente proveniente de um processo de bioremediação de AMD, contendo sulfureto gerado biologicamente, pode ser eficientemente utilizado na recuperação de ouro de soluções aquosas, obtendo-se NPs de ouro metálico. Este mesmo efluente permite ainda sintetizar NPs Ag_2S e o nanocompósito Ag_2S/TiO_2, com propriedades semicondutoras. Todas a partículas recuperadas nas diversas etapas do trabalho podem ser utilizadas nas aplicações já conhecidas para as mesmas, ou testadas para outros fins.

Palavras-chave: Metais Preciosos, Metais do Grupo da Platina, Extracção por Solventes, Bioremoção/Biorecuperação, Nanopartículas

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List of Abbreviations

AAS - atomic absorption spectrometry

ARDRA - amplified ribosomal DNA restriction analysis

AMD - acid mine drainage

ATP - adenosine triphosphate

Cyanex 302 - bis(2,2,4-trimethylpentyl)monothiophosphinic acid
Cyanex 921 - tri-octyl phosphine oxide
D - distribution coefficients
DCE – dichloroethano
DCM - dichloromethano
DGGE - denaturing gradient gel electrophoresis
DMDCHMA - *N,N'*-dimethyl-*N,N'*-dicyclohexylmalonamide
DMDCHTDMA - *N,N'*-dimethyl-*N,N'*-dicyclohexyltetradecylmalonamide
DMDPHMA - *N,N'*-dimethyl-*N,N'*-diphenylmalonamide
DMDPHTDMA - *N,N'*-dimethyl-*N,N'*-diphenyltetradecylmalonamide
DMDCHSA - *N,N'*-dimethyl-*N,N'*-dicyclohexylsuccinamide
DMDPHSA - *N,N'*-dimethyl-*N,N'*-diphenylsuccinamide
DNA - deoxyribonucleic acid
DR – diffuse reflectance
DRS - diffuse reflectance spectroscopy
EDS or EDX - energy dispersive X-ray
Eh – redox potential
ESI - electro-spray
FTIR – Fourier Transform Infrared Spectroscopy
GC – Gas Chromatoghrapy
HPLC - High Performance Liquid Chromatography
IX – Ion Exchange
LC-MS - Liquid Chromatography–Mass Spectrometry
LLE – Liquid-liquid Extraction
MP – Metabolic Products
NMR - Nuclear magnetic resonance
NPs – Nanoparticles
PBDEs - Polybrominated diphenyl ethers
PC-88A - 2-ethylhexyl phosphonic acid mono-2-ethylhexyl ester
PCB - Printed Circuit Board
PCR - Polymerase Chain Reaction
PGM - Platinum Group Metals
rRNA - Ribosomal ribonucleic acid
RFLP - Restriction fragment length polymorphism analysis
SACC - Spent Automobile Catalytic Converters
SRB – Sulphate-Reducing Bacteria

SRS - Stanford Research Systems

SSCP - single-stranded conformation polymorphism

SX – Solvent Extraction

TAE - Tris-acetate-EDTA

TBP - tributyl phosphate

TCE – Trichloroethylene

TGGE - temperature gradient gel electrophoresis

TLC - Thin-Layer Chromatography

WEEE - Waste Electrical and Electronic Equipment

XRD - X-Ray Diffraction

%E – extraction percentage

%S – stripping percentage

CHAPTER 1

General Introduction

1. INTRODUCTION

Precious metals, namely those from Platinum Group Metals (PGM) are of great interest due to their high economic value and properties. The demand of these metals is increasing mostly because of the wide range of industrial applications, namely as catalysts in organic processes, as value added components in metal alloys and vehicle catalytic converter systems. In addition, PGM have equally been used in chemical, pharmaceutical, petroleum and electronic industries and in the industry of jewellery (Cieszynska and Wisniewski, 2011). At the same time, the synthesis of metallic nanoparticles (NPs) has also been an object of increased interest due to their unusual chemical, optical, electronic and magnetic properties. Metallic NPs have a high surface/volume ratio due to their small size and high proportion of edges and corners and therefore are useful in applications such as catalysis, fuel cells, biosensing, biological labelling, electronics, optical devices and in the control of drug delivery (Legke *et al.*, 2007). In particular, the extensive use of NP of precious and PGM in industrial and automotive catalysts is increasing (Lloyd *et al.*, 2005). Moreover, the superior catalytic activity of various metallic NPs has led to a widespread interest of their use for the removal of various environmental contaminants, including chlorinated compounds (Baxter-Plant *et al.*, 2003) and hexavalent chromium (Mabbett *et al.*, 2004; Omole *et al.*, 2007).

As the result of their rising demand and of the progressive depletion of the primary raw material resources, there is a growing attention in the recovery and reutilization of PGM from industrial wastes and their possible recovery as metallic NPs is of special interest. Nowadays, the European Waste Electrical and Electronic Equipment (WEEE) Directive requires that the PGM contained in the electrical and electronic equipment be recovered and recycled. As an example, every year about 20 to 50 million tons of WEEE, known as e-waste, is reported to be generated in the world (Heart, 2009). The UK produces around 50,000 tons of printed circuit board (PCB) scrap per year, with only 15% being recycled (Pilone and Kelsall, 2006). PCB are about 3% of weight of the total amount of WEEE and it contains nearly 28% metals and almost 70% non-metallic materials (Sohaili *et al.*, 2012).

Reprocessing of PGM via traditional hydro- or pyrometallurgical routes is costly and not environmentally friendly, since they also generate gases and liquors containing residual precious and PGM. Therefore, it is urgent to invest on processes that could provide some value-adding for the treatment of effluents

(Silva et al, 2005) or that could turn viable the recycling of different sorts of materials containing PGM (Malik and Paiva, 2010).

A few years ago, some authors have been investigating the use of N,N'-tetrasubstituted malonamide derivatives in the recovery of metal ions from hydrochloric acid solutions by liquid-liquid extraction (Malik and Paiva, 2010; Costa et al, 2003; Costa *et al.*, 2007; Malik and Paiva, 2008; Malik and Paiva, 2009; Costa *et al.*, 2013). These extractants, containing carbon, hydrogen, nitrogen and oxygen atoms only, were chosen to be studied due to its better environmental-friendly character comparing to the phosphorus compounds, which are the most popular in the hydrometallurgical industry (Rydberg *et al.*, 2004). A number of N,N'-tetrasubstituted malonamide derivatives with slight different structural details were already synthesized and studied for the selective separation of PGM (Malik and Paiva, 2010; Malik and Paiva 2008; Malik and Paiva, 2009) and as iron(III) extractants (Costa *et al.*, 2003; Costa *et al.*, 2007). Malik and Paiva (2010) demonstrated that N,N'-tetrasubstituted malonamide derivative is only effective to the mutual separation of the three PGM tested (Pt, Pd and Rh) and for their selective separation from base metals, if tin(II) chloride is added to the feed leaching chloride solutions.

Another approach that has been investigated for metals removal is the biorecovery strategies using microorganisms (Konishi *et al.*, 2007). In fact, they have been considered one of the most environmentally safe and low cost technology. The microorganisms can remove metal ions from aqueous solutions through, adsorption, precipitation or reduction reactions (Konishi *et al.*, 2007), leading, in many cases, to the formation of metallic NPs (Mandal *et al.*, 2005).

Noble metals, such as, silver (I) and gold (III) have been biologically reduced and deposited as NPs using microorganism (Klaus *et al.*, 1999; Kashefi *et al.*, 2001; Sastry *et al.*, 2003; Lloyd, 2003; Lloyd *et al.*, 2003; Schröfel and Kratošová, 2011; Binupriya *et al.*, 2010; Cai *et al.*, 2011). However, in spite of several reported studies about the bioremoval of metals from solutions, the mechanisms involved are not normally well understood.

In this work both chemical and biological approaches were applied for precious metals removal and recovery. In the chemical approach novel extractants were synthetized and their capacity for PGM removal in solvent extraction processes was tested. In the biologic approach the capabilities of anaerobic bacteria communities for the recovery of precious metals (gold, silver, platinum and palladium) as NPs was investigated. It is expected that this work will contribute for the development of clean, low cost and environmentally acceptable procedures aiming precious metals removal/recovery from secondary sources.

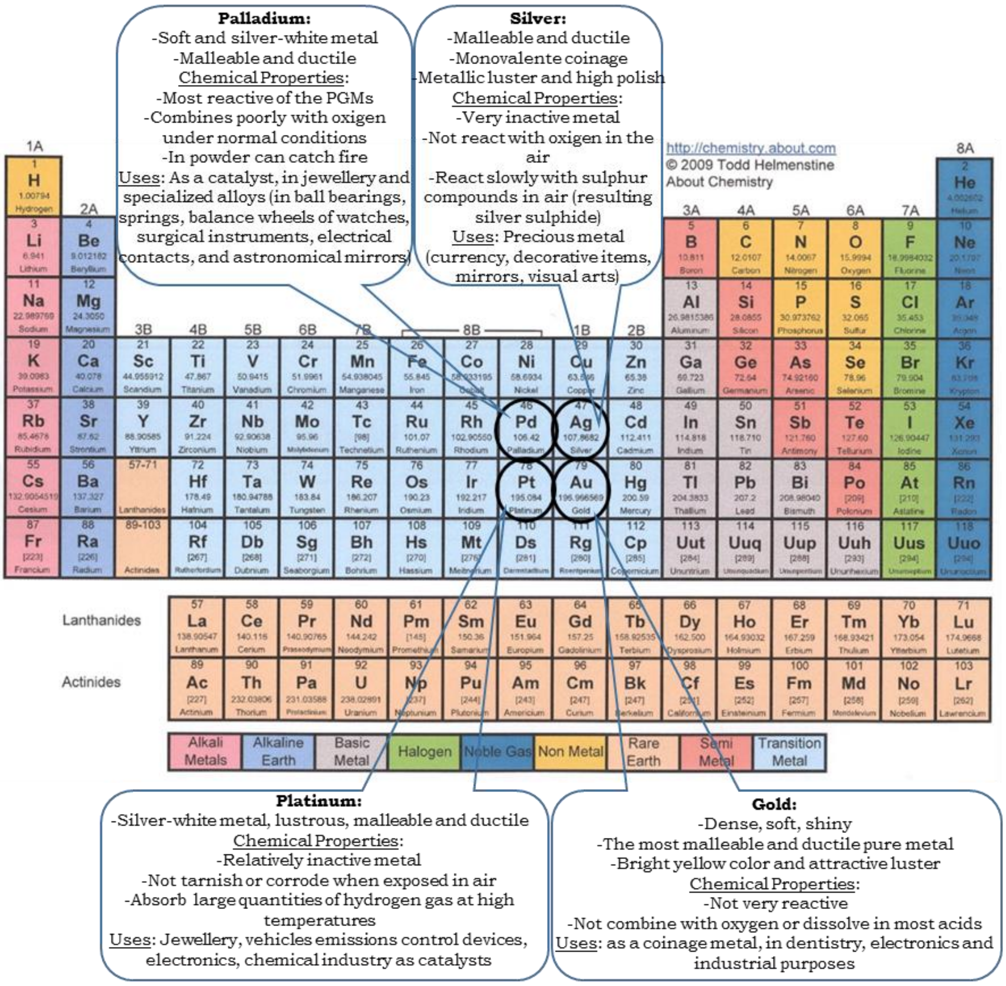
1.1. Precious Metals

Metals were classified by Lavoisier as elements that are "metallic, oxidizable and capable of neutralizing an acid to form a salt". He included on that category, antimony and arsenic (which are not considered metals today), silver, bismuth, cobalt, copper, tin, iron, manganese, mercury, molybdenum, nickel, gold, platinum, lead, tungsten, and zinc. Nowadays, the metals classification is different, being usually classified in three categories: toxic metals (e.g., Cr, Zn, Cu, Ni, Cd, As, etc.), precious metals (e.g., Pd, Pt, Ag, Au, etc.) and radionuclides (e.g., U, Th, Ra, Am, etc.) (Bishop, 2002; Wang and Chen, 2006). Metals are distributed all over the world and constitute about 75% of the known elements (Raab and Feldmann, 2003; Gadd 2010).

Precious metals are stable elements in air and do not corrode, independently of their aggregation state. The precious metals include, gold (Au) and PGM which comprises platinum (Pt), palladium (Pd) ruthenium (Ru), iridium (Ir) and rhodium (Rh). Osmium (Os) is also defined as a PGM, however, their high toxicity at certain temperatures makes it not much used. It is not consensual that silver (Ag) is a precious metal, some literature considered to be a semi-precious metal, because it is affected by sulphur (Baltzer, 2007).

The precious metals gold, silver, platinum, and palladium have been historically valued for their beauty and rarity, which confers a high economic value to these metals. Platinum and gold are about 80 times more costly than silver and the price of platinum usually has higher than gold (World of Earth Science, 2003).

The Periodic Table of the Elements is shown in Figure 1-1 and the metals used in the present work are identified with circles.



Palladium:
-Soft and silver-white metal
-Malleable and ductile
Chemical Properties:
-Most reactive of the PGMs
-Combines poorly with oxigen under normal conditions
-In powder can catch fire
Uses: As a catalyst, in jewellery and specialized alloys (in ball bearings, springs, balance wheels of watches, surgical instruments, electrical contacts, and astronomical mirrors)

Silver:
-Malleable and ductile
-Monovalente coinage
-Metallic luster and high polish
Chemical Properties:
-Very inactive metal
-Not react with oxigen in the air
-React slowly with sulphur compounds in air (resulting silver sulphide)
Uses: Precious metal (currency, decorative items, mirrors, visual arts)

Platinum:
-Silver-white metal, lustrous, malleable and ductile
Chemical Properties:
-Relatively inactive metal
-Not tarnish or corrode when exposed in air
-Absorb large quantities of hydrogen gas at high temperatures
Uses: Jewellery, vehicles emissions control devices, electronics, chemical industry as catalysts

Gold:
-Dense, soft, shiny
-The most malleable and ductile pure metal
-Bright yellow color and attractive luster
Chemical Properties:
-Not very reactive
-Not combine with oxygen or dissolve in most acids
Uses: as a coinage metal, in dentistry, electronics and industrial purposes

Figure 1-1 – Periodic table of elements. The black circle identify the precious metals studied in the present work and in the blue balloons are several characteristics of each one of those metals

The increasing application of metals contributes for the metals pollution, which is a serious environmental problem. Furthermore, metals are a resource that is becoming rare, especially the precious metals. Thus, it is very important to recover them from secondary sources and from metal-bearing wastewaters.

1.1.1. Gold

In each ton of rock there are only about eight pennies of gold, which makes gold so rare. In geologic processes, for the occurrence of gold is necessary that this metal is concentrated at least 250 times the value found in average rock. Even at this concentration, there is still one million times more rock than gold and the gold is rarely seen (World of Earth Science, 2003).

Gold is resistant to corrosion by air and by most chemicals, and can only be dissolved in a mixture of nitric and hydrochloric acids, a solution called aqua regia. Thus, gold has been mentioned as virtual indestructible which means that almost all gold ever mined could still be in use today (World of Earth Science, 2003).

Nowadays, it is estimated that around 2,000 tons of gold are added per year to the total gold extracted, indicating that probably 70% of all gold recovered has been mined in this century. Nevada is the leading gold producing state in the U.S.A and the Republic of South Africa is the world's leading gold-producing nation.

This fascinating and exceptional precious metal has several characteristics which makes it unique, such as, the low toxicity and the singular beauty being a treasured because of its colour (Mata *et al.*, 2009; Syed, 2012). Some basic gold properties are shown below in Table 1-1. Their unique physical and chemical properties, confer to gold an extraordinary ductile and malleable metal and thus gold is still a cosmic demand in jewellery, high-tech industries and medical applications (Ramesh *et al.*, 2008). The excellent electrical conductivity, low contact electrical resistance for inserting connections and outstanding corrosion resistance, encouraged in the last decades, their application in electronic and electrical industries (Baba, 1987). Therefore, the increasing demand for gold requires the gold's recovery from the inevitably growing waste products (Syed, 2006) and its recovery is also interesting due to its characteristics and high market prices (Mata *et al.*, 2009; Syed, 2012).

1.1.2. Silver

Silver is characterized by its silvery colour, brightness, malleable, ductile and presents a great electric conductivity. This metal has a low reactivity and is highly resistant to corrosion. Some basic silver properties are shown below in table 1. Silver is not nearly as precious, dense, or noble as gold or platinum. This metal can be found in the native state and in combination with sulphur, as in the mineral argentita (Ag_2S). Once silver is more reactive than gold, can be leached from surface rocks and carried downward in solution. This process, called supergene enrichment, can concentrate silver into exceedingly rich deposits at depth. Mexico has traditionally been the world's leading silver producing country, however nowadays the United States, Canada, and Peru contribute with significant amounts. The silver reserves worldwide in 2014 was estimated of approximately 25,000 metric tons in the United States and Australia with an estimated 85 thousand metric tons, was ranked second worldwide based on silver reserves (http://www.statista.com/statistics/273649/silver-reserves-of-countries/, accessed in 23/12/2015).

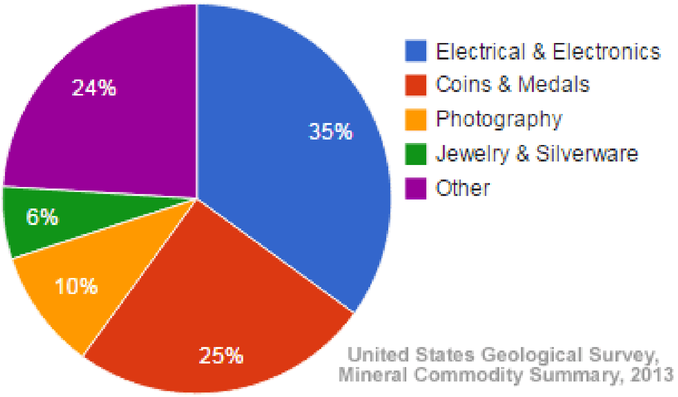
Table 1-1 – Basic properties of Gold and Silver

Basic Gold ans Silver Properties					
Name	**Chemical Symbol**	**Oxidation states**	**Highest purity available (%)**	**Melting Point (°C)**	**Density (g/cm^3)**
Gold	Au	-1, **+1**, +2, **+3**, +5	99.998	1064	19.32
Silver	Ag	-2, -1, **+1**, **+2**, +3, +4	99.990	962	10.49

NOTE: Oxidation states in bold are the common ones and the other oxidation states exist but are rarer.

Hydrothermal veins constitute the most important source of silver. The extraction as soluble cyano's complexes may occur starting from their ores.

Although significant quantities of silver are still used in jewellery, silver ware, and coinage, larger amounts are consumed in new applications, such as, photographic and electronics industries (Figure 1-2)(Ferré, 2015).



United States Geological Survey, Mineral Commodity Summary, 2013

Figure 1-2 - Distribution of the amount of silver by category of use, in United States. This data is from the United States Geological Survey. The category “Other” is responsible for almost a quarter of the silver used and is fragmented into hundreds of different uses (from Ferré, 2015).

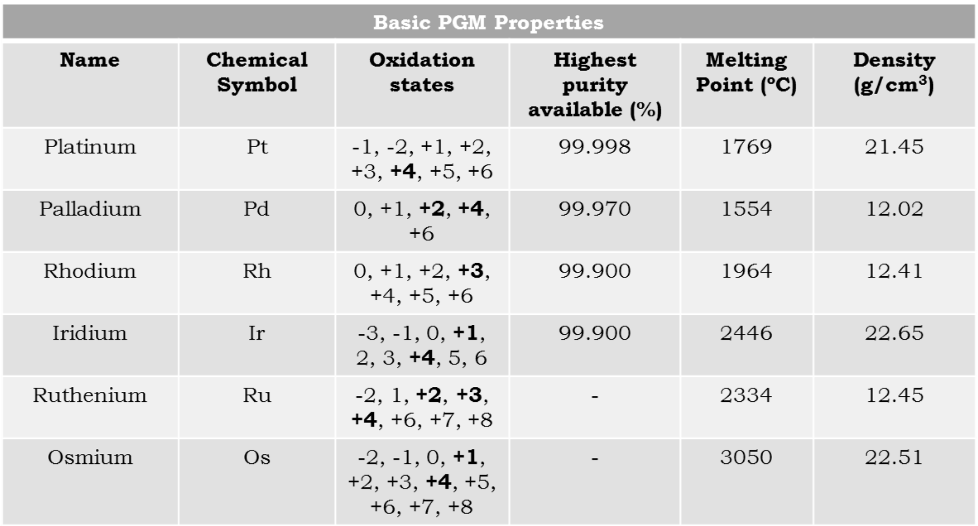
The incomparable thermal and electrical conductivity of silver comparing with other metals implies that this metal cannot easily be replaced by less expensive materials. As mentioned before, silver is very important due to its applications, thus, its recovery from secondary sources has been attempted. Recently, a considerable quantity of metal has been obtained by recycling, especially by the photographic industry (Souza *et al.*, 2013).

1.1.3. Platinum Group Metals (PGM)

PGM comprises six closely related metals: platinum (Pt), palladium (Pd), rhodium (Rh), ruthenium (Ru), iridium (Ir) and osmium (Os), which occurs commonly together in nature and are rare in the Earth's crust. Almost all of the world’s supply of PGM (around 90%) is extracted from deposits in South Africa, Russia, Canada and USA, whereas the major consumers of PGM (roughly 80%) are the North America, Europe, and Japan (Lloyd *et al.*, 2005).

The PGM are characterized by high corrosion resistance, stability to oxidation at high temperatures and by their exceptional catalytic properties. Some PGM basic properties are shown in Table 1-2.

Table 1-2 – Several basic properties of PGM



Basic PGM Properties

Name	Chemical Symbol	Oxidation states	Highest purity available (%)	Melting Point (ºC)	Density (g/cm^3)
Platinum	Pt	-1, -2, +1, +2, +3, **+4**, +5, +6	99.998	1769	21.45
Palladium	Pd	0, +1, **+2**, **+4**, +6	99.970	1554	12.02
Rhodium	Rh	0, +1, +2, **+3**, +4, +5, +6	99.900	1964	12.41
Iridium	Ir	-3, -1, 0, **+1**, 2, 3, **+4**, 5, 6	99.900	2446	22.65
Ruthenium	Ru	-2, 1, **+2**, **+3**, **+4**, +6, +7, +8	-	2334	12.45
Osmium	Os	-2, -1, 0, **+1**, +2, +3, **+4**, +5, +6, +7, +8	-	3050	22.51

NOTE: Oxidation states in bold are the common ones and the other oxidation states exist but are rarer.

However, to use PGM metals complex extraction and refining processes are required. For instance, for mining and production of platinum the process is very time-consuming because the amount of this metal processed in the ore has to undergo extraction, concentration and refining stages (Jonck, 2008). PGM availability has become more and more limited which have contributed for the raise of PGM's economic value.

Platinum

Platinum, is a silver-white metal and is found in pure metallic chunks in stream placers, like gold, and the average crustal abundance of platinum is comparable to that of gold. Platinum is chemically inert even at high temperature, and presents a melting point of 1769°C. The world's largest producer of platinum is South Africa, with vast platinum ore deposits in the Merensky Reef of the Bushveld complex, followed by Russia (Xiao and Laplante, 2004; George, 2007). Platinum can be produced commercially as a by-product of nickel ore processing, corresponding to two parts per million of the ore. Hence, the principal raw materials are concentrates produced from ores, mattes and slimes from nickel and copper operations. However, the secondary materials are also an important source. These secondary sources can be spent chemical and auto exhaust catalysts, and electronic and electrical component scrap.

The world platinum demand estimated for 2012 was 0.470 million ounces (Figure 1-3). In 2012, it was estimate a total world platinum production of 5.8 million ounce and estimates a double digit decline in recycling supply. The investment demand for platinum has been basically stagnant for the last 6 years (Smithmier, 2013).

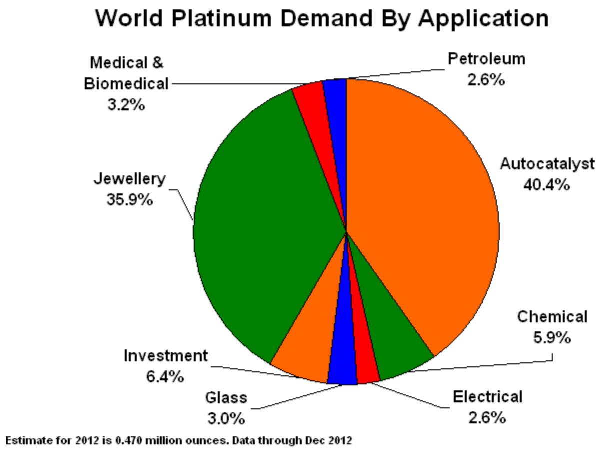


Figure 1-3 – World Platinum Demand by application (from Smithmier, 2013, in http://hightowerreport.com/2013/02/major-move-in-platinum-overnight-2013-02-04/)

The demand for gold and platinum are expected to continue to increase because of their rarity and unique properties. Silver is more closely tied to industry, and the demand for silver is expected to rise and fall depending on economic conditions.

Palladium

Palladium occurs on earth with a relative abundance of 0.0006 parts per million. The major sources of palladium were found in Russia and South Africa. In North America, palladium was found with platinum and other PGM in Montana where it is now extracted by the Stillwater Mining Company (Palladium, Metal of the 21st Century, 2013). However, palladium is commercially produced from nickel-copper deposits in South Africa and Ontario, Canada (Chevalier, 2008).

Palladium has unique chemical and physical properties that allow its use in many industrial sectors, such as, chemical and petroleum industry, production of automobile catalysts, electronic devices, dental applications, and fine jewellery. In the past 20 years, the use of Pd in these industries increased, which resulted in higher environmental levels of this metal.

Palladium has been shown to be toxic, among metals, Pd ions are one of the most frequent reacting sensitizers. The exposure to palladium can cause acute toxicity or hypersensitivity with respiratory symptoms, urticaria and, less frequently, contact dermatitis (Goossens *et al.*, 2006).

For all these reasons, it is extremely important the development of new processes that could provide some value-adding for the treatment of effluents, or that can turn viable the recycling of different classes of materials containing PGM in the final period of their lives.

1.2. Wastes containing precious metals

In the last years, it has been observed an increasing demand for precious metals, namely PGM, due to the wide range of industrial applications, already mentioned (Cieszynska and Wisniewski, 2011). Their wide use generates a considerable amount of wastes (secondary sources) which present a content of precious metals far higher than their content in natural ores.

In natural ores, the average concentration of precious metals is around 1-30 ppm, while in secondary sources is about 1-2000 ppm (Won *et al.*, 2014). It was assigned the name of urban mines of precious metals' to the spent catalysts, electronic wastes and other secondary sources of precious metals. There are three secondary sources of precious metals: atmospheric air, solid wastes and liquid wastes.

Some of the secondary sources from liquid wastes are (Won *et al.*, 2014):

- Effluents from precious metals as catalyst in manufacturing processes

- Effluents of automotive catalyst waste recycle industries
- Effluents of precious metal refineries
- Effluents of the WEEE and/or PCB refinery industries
- Jewellery processing/making industry waste waters
- Municipal sewage
- Hospital sewage
- Run off street water due to rain

1.3. Chemical methods used to remove precious metals from aqueous solutions

The recovery of precious metals from effluents is very important from both economic and environmental points of view. Hydro- and pyrometallurgical processes have been presented as conventional recycling techniques. Nevertheless, these processes present several disadvantages, such as, significant investments, labour and time are required and the processes also require chemicals which generate a secondary waste (Jacobsen, 2005). In the past years, the studies focused on the separation, concentration and purification of aqueous solutions containing mixtures of Pt, Pd and Rh have been increasing. Therefore, several relevant studies aiming the recovery of these three PGM from chloride solutions are highlighted namely liquid-liquid extraction and ion exchange (IX).

1.3.1. Liquid-liquid Extraction

Liquid-liquid extraction, also called solvent extraction (SX), is defined as a method for separating components of a solution by using an unequal distribution of the components between two immiscible liquid phases.

In general, SX is performed by mixing two immiscible phases (aqueous and organic), allowing the selectively transfer of solutes from one phase to the other and then enabling the separation of the two phases. Commonly, one phase is the aqueous solution that contains the components to be separated, and the other phase is an organic solvent which has high affinity for some specific components of the solution. These process is reversible, so the solvent loaded with solute(s) can be placed in contact with another immiscible phase that has a higher affinity for the solute than the organic phase. The first step of solute transference from one phase into the solvent phase is mentioned as extraction and the second step of the transference of the solute from the solvent back to the second (aqueous) phase is referred as stripping or back-extraction (Law and Todd, 2008). A general scheme of this process is shown in Figure 1-4.

The difference of densities between both phases allows these two immiscible fluids to have the capacity on rapidly separating after being mixed together. (Law and Todd, 2008). The extractant or reagent is generally dissolved in an organic diluent (organic phase).

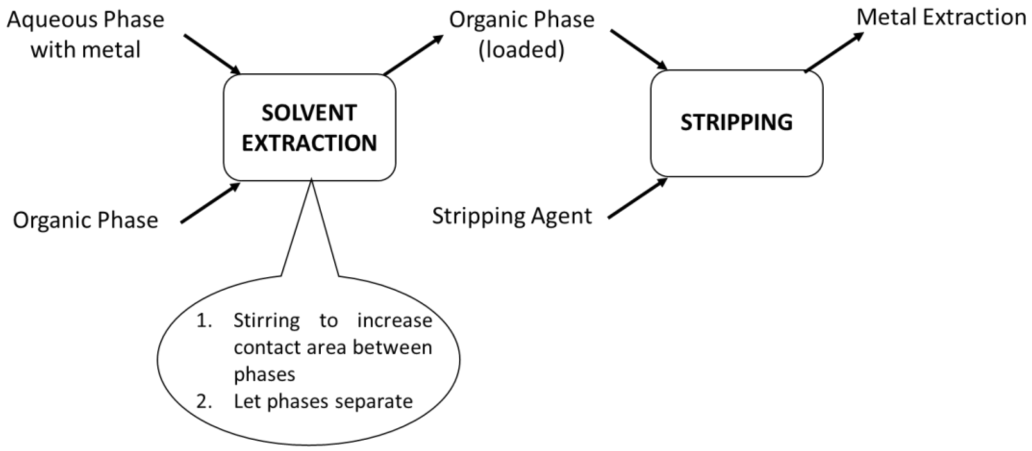


Figure 1-4 – Procedure of a Solvent Extraction Experiment

Solvent extraction is a heterogeneous chemical process in which the reaction involved in the transfer of metal occurs on the aqueous organic interface to the final equilibrium concentrations of the reaction product, and is accompanied by the distribution of product complexes between two immiscible phases. The equilibrium constant is related to the extent of the reaction for solute–ligand system. The analyte, (metal ions, cations, and anions) present in the aqueous phase are extracted, through reversible chemical reactions, into the organic phase and an organic-soluble neutral complexes are formed. In general, the extractant agent, has very low solubility in the aqueous phase, however interacts with metal ions and have a high solubility in the organic phase.

In SX experiment, the extraction may be complete or incomplete as show in Figure 1-5. In an incomplete extraction, just part of the analyte present in the aqueous phase is extracted to the organic phase (Figure 1-5 A), while in a complete extraction all the analyte is extracted (Figure 1-5 B).

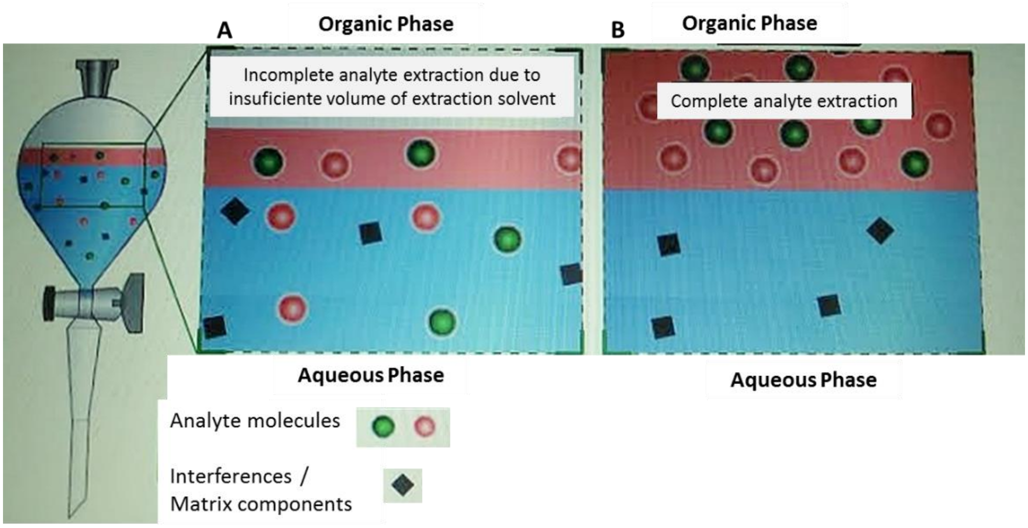


Figure 1-5 – Solvent extraction: **A** – incomplete analyte extractiom, **B** – Complete analyte extraction (adapted from http://www.chromacademy.com/lms/sco59/Sample_Preparation_Liquid-Liquid_Extraction_Techniques.pdf)

Some extractants have been used for Pd and Pt extraction, such as, triisooctylamine (Hostarex A324) and triisobutylphosphine sulphide (Cyanex 471X) (Nowottny *et al.*, 1997). The hydroxyquinoline derivative (Kelex 100) in the presence of tin(II) chloride has also been studied for the separation of Pt, Pd and Rh (Alam and Inoue, 1997) and Superlig® series resins have been applied to PGM separation by ion exchange (IX) (Izatt *et al.*, 1999). Cyanex 921 (Mhaske and Dhadke, 2001a) or Cyanex 925 (Mhaske and Dhadke, 2001b) are phosphine oxides that were mentioned as unique extractants for Pt, Pd and Rh separation and recovery from simulated spent automobile catalytic converters (SACC) leaching solutions. The absence or presence of tin(II) chloride proved to be an important factor in the complete separation of the PGM (Costa *et al.*, 2013; Malik and Paiva, 2009). Silica based (poly)amine ion exchangers have also been successfully applied to the selective removal of PGM from industrial metal refinery effluents containing large amounts of Ni, Cu and Fe (Kramer *et al.*, 2002; Kramer *et al.*, 2004); achieving a quantitative Pt and Pd removal and a low Rh recovery. Rh proved to be difficult to separate from the other two PGM by SX, thus some authors proposed a chromatographic method involving hydrophilic gels to separate the metal ions by differential migration. Surfactant gel extraction by cetylpyridinium chloride has also been found adequate to separate the three PGM (Murakami *et al.*, 2008), however with a low HCl concentration (0.001M) in the aqueous solutions.

In recent years, some thiapentanamides and thiodiglycolamides have been reported as good extractants for Pd, Pt and Au (Narita *et al.*, 2008) and dialkyl sulphoxides were used to extract some PGM from 1-6M HCl solutions, with the following order results, Pd(II)>Pt(II)>Ir(IV)>Pt(IV)>Rh(III)>Ir(III).

Pd has been efficiently and selectively extracted from simulated SACC solutions containing 0.1 g/L each of Pt, Rh, Cu, Fe, Zn and Ni in 0.2-7M HCl solutions by a sulphide-containing monoamide (Narita *et al.*, 2009) and dibutylsulfoxide showed to be effective in the separation of Pd from Pt (Pan and Zhang, 2009).

Sudies with malonamide derivatives, as N,N' - dimethyl-N,N'-diphenyltetradecylmalonamide (DMDPHTDMA) were carried out and a successful extraction of Rh (Malik and Paiva, 2008) and Pt (Malik and Paiva, 2009) from chloride media were obtained.

The inherent advantages of N,N'-tetrasubstituted malonamide derivatives comparing to phosphorus extractants, currently used in nuclear industry, makes the first ones advantageous to be used. The malonamide derivatives usually exhibit good selectivity, fast kinetics, high efficiency and, most of all, complete incinerability (Thiollet and Musikas, 1989) and have also been explored for the extraction of Fe(III) from concentrated chloride media (Costa *et al.*, 2003; Costa *et al.*, 2007). The mutual separation of Pt, Pd and Rh by DMDPHTDMA and their selective extraction over some associated elements was also exploited (Malik and Paiva, 2010).

Other example of diamide extractants are the N,N´-tetrasubstituted succinamides, which was recently tested for the extraction of iron(III) (Cui *et al.*, 2015). The advantage that succinamides may have relatively to malonamides for Pt(IV) extraction is the higher distance between the carbonyl groups of these molecules, due to the existence of an additional carbon atom placed between them, which could theoretically be a structure more suitable to "accommodate" the high sized platinum extracted species. This is also supported by the evidence that an ionic pair mechanism is probably responsible for extraction of Pt(IV) by N,N´-tetrasubstituted malonamides. This ionic pair is eventually involved in the oxygen protonation of the carbonyl group (Paiva *et al.*, 2014).

The chemical structures of some malonamide and succinamide derivates are represented in Figure 1-6.

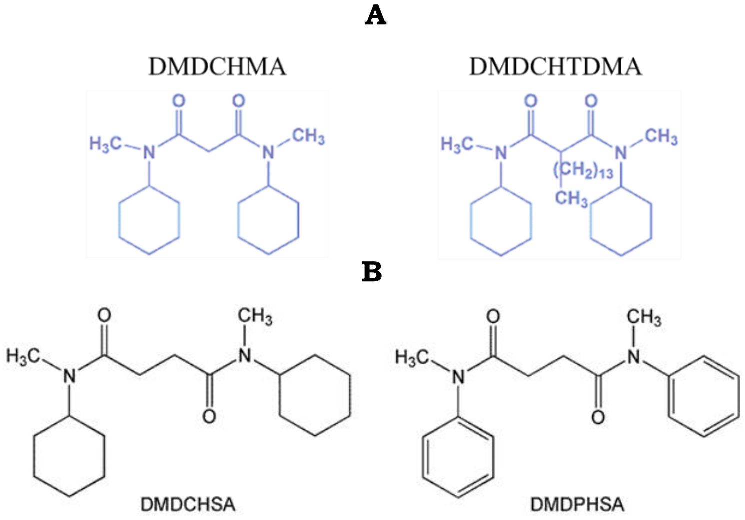


Figure 1-6 - Malonamide and succinamide derivates synthesied and tested in the present work. The malonamides derivates (A) are N,N'-Dimethyl-N,N'-dicyclohexylmalonamide (DMDCHMA) and N,N'-dimethyl-N,N'-diphenyltetradecylmalonamide (DMDPHTDMA), while the succinamide derivates (B) are N,N'-dicyclohexyl-N,N'-dimethylsuccinamide (DMDCHSA) and N,N'-dimethyl-N,N'-diphenylsuccinamide (DMDPHSA).

1.3.2. Alternative chemical methods

The removal of metals from effluents can be performed by several conventional processes, namely chemical precipitation, ion exchange (IX), electrochemical and sorption methods (Barakat, 2011).

The principal technique used for heavy metal removal from effluent is chemical precipitation, based on the use of alkaline agents. The metal removal mechanism is presented in Eq. (1) (Wang *et al.*, 2004):

$$M^{2+} + 2(OH)^{-} \leftrightarrow M(OH)_2\downarrow \qquad \text{Eq. (1)}$$

where M^{2+} represents the dissolved metal ion and OH^{-} the precipitating agent, while $M(OH)_2$ is the insoluble metal hydroxide. The adjustment of the pH to basic conditions (pH 9–11), generally improves significantly the removal of heavy metal by chemical precipitation (Barakat, 2011), but the minimum required pH depends on the hydroxide metal solubility. The use of other chemical precipitants such as sulphide, hidroxides and phosphate, chemically or even biologically generated, can also be employed (Fu and Wang, 2011).

In the last years, IX process has also been used for heavy and precious metals removal and/or recovery. IX is a versatile separation process and has a wide range of applications in the field of water and wastewater treatment (Al-Enezi *et al.*, 2004). This method is based on the exchange between the substrate and surrounding medium, which may be two electrolytes or an electrolyte solution and a complex. The IX reaction mostly used is reversible and in these cases the ion exchanger can be reused many times (Hubicki and Kołodyńska, 2012). The most used ion exchangers are ion exchange resins (functionalized porous or gel polymer), however others ion exchangers can be used, as zeolites, montmorillonite, clay, and soil humus. Some advantages of IX are the recovery of metal value, selectivity, less sludge volume produced and the meeting of strict discharge specifications (Zewail and Yousef, 2015).

Electrochemical methods have also been proposed for metals removal. They are based on the plating-out of metal ions on a cathode surface, by the application of a suitable potential and allow the recover of metals in the elemental state, which is an important advantage. However, this technology has not been widely applied due to the relatively high capital investment and expensive electricity supply (Fu and Wang, 2011).

All these processes present disadvantages, such as, incomplete removal, high-energy requirements, and production of high volumes of toxic and relatively soluble sludge (Eccles, 1999), that requires disposal. Thus, alternative numerous approaches have been studied. Adsorption has been mentioned as a possible treatment and although the use of activated carbon is quite commum, the search for other low-cost adsorbents with metal-binding capacities has been intensified (Leung *et al.*, 2000). However, the development of "green technologies", considered low-cost and environmentally desirable (that can be rely on the use of wastes or biomass), to overcome these disadvantages has also gained focus.

1.4. Biological methods used to remove precious metals from aqueous solutions

The biologic methods, based on the use of live or dead biomasss, or on the utilization of products generated by organisms, are usually nontoxic, high-yield and energy saving (occur under normal air pressure and temperature). Hence, bioprocesses mediated by living organisms (employing their cells, enzymes, transport chains, etc.) or even their dead cels have been developed for metallic NPs synthesis (Schröfel and Kratošová, 2011), which is an advantage considering the wide applications of these NPs. Several organisms have been used for metals removal, such as, plants, algae, fungi, yeasts and bacteria (Fu and Wang, 2011). Many unicellular and multicellular organisms are known to produce intracellular or extracellular metallic NPs (Thakkar *et al.* 2010) and specially the bioremediation ability of some bacteria have gained focus. For example, the immobilization of metallic NPs on bacterial surfaces has been reported (Deplanche *et al.*, 2011; De Corte *et al.*, 2011). These NPs have a high surface area and reactivity, and the bacterial cells work as a support/matrix for the NP, which is recoverable and simultaneously reduce the potential environmental and health risks associated with handling free NPs (Bennett *et al.*, 2013).

Technologies using sulphate-reducing bacteria (SRB) have been developed to treat heavy metal contaminated effluents with success (Rashamuse and Whiteley, 2007). The SRB are known as economical, environmental and biotechnological important organisms, once they reduce metallic ion species even with the involvement of microbial enzymes in this process (Mikheenko *et al.*, 2008). Some authors have demonstrated the use of SRB in PGM removal from aqueous solutions. For example, the bioreductive deposition of Pd(0) onto biomass of D. *desulphuricans* (Lloyd *et al.*, 1998; Yong *et al.*, 2002a; Yong, *et al.*, 2002b), the bioaccumulation of Pd by a wild-type of *Desulfovibrio fructosivorans* (Mikheenko *et al.*, 2008) and the biosorption ability showed by *Desulfovibrio desulfuricans* to Pd and Pt (Vargas *et al.*, 2004) have been extensively investigated.

A few studies have reported the reduction of platinum (IV) to Pt(0) NPs, by biological methods. The bioreduction of platinum ions to Pt(0) NPs have been achieved with several pure cultures, some of them already mentioned before, including *Shewanella algae* (Konishi *et al.*, 2007), cyanobacteria (Lengke *et al.*, 2006; Brayner *et al.*, 2007), *Desulfovibrio sp.* (Rashamuse and Whiteley, 2007; Rashamuse *et al.*, 2008) and also by communities, namely SRB consortia (Riddin *et al.*, 2010).

The bioaccumulation of gold by two diatoms has been reported by Chakraborty and colleagues (2006). Gold can also be recovered by biosorption and bioreduction processes with a brown alga *Fucus vesiculosus* (Mata *et al.*, 2009).

Thus, it can be concluded that live algal biomass may be a viable and cost effective way for gold biorecovery.

The bioaccumulation of silver ions Ag(I), from the solution or wastewater, on gilled macrofungus *Pleurotus platypus* was studied (Das *et al.*, 2010) and showed to be a modern and innovative approach for the study of interactions between biomass and metal ions.

1.4.1. The case of SRB

SRB are anaerobic microorganisms characterised by using sulphate as a terminal electron acceptor in the degradation of organic compounds. These bacteria played an important role in both sulphur and carbon cycles and are ubiquitous in this anoxic habitats (Muyzer and Stams, 2008).

Some years ago, SRB was applied in acid mine waters (Colmer and Hinkle, 1947) for remediation of sulphate and metal-containing wastewater (Tuttle *et al.*, 1969). The combination of the simultaneous sulphate and metals removal contributes to the fact that SRB-based processes be considered the most promising alternative for the treatment of several kinds of industrial wastewaters, namely acid mine drainage (AMD) (Johnson and Hallberg 2005; Neculita *et al.* 2007). SRB can be found in extreme habitats (for example, with extreme pH), such as mining wastewaters, with pH near 2 and in soda lakes, with pH around 10 (Muyzer and Stams, 2008). These bacteria can also be present in aquifers and in engineered systems, like anaerobic wastewater treatment plants (Muyzer and Stams, 2008; Barton and Fauque, 2009).

The sulphate reduction process may occur by assimilatory or dissimilatory pathways (Grein *et al.*, 2012), as demonstrated in Figure 1-7.

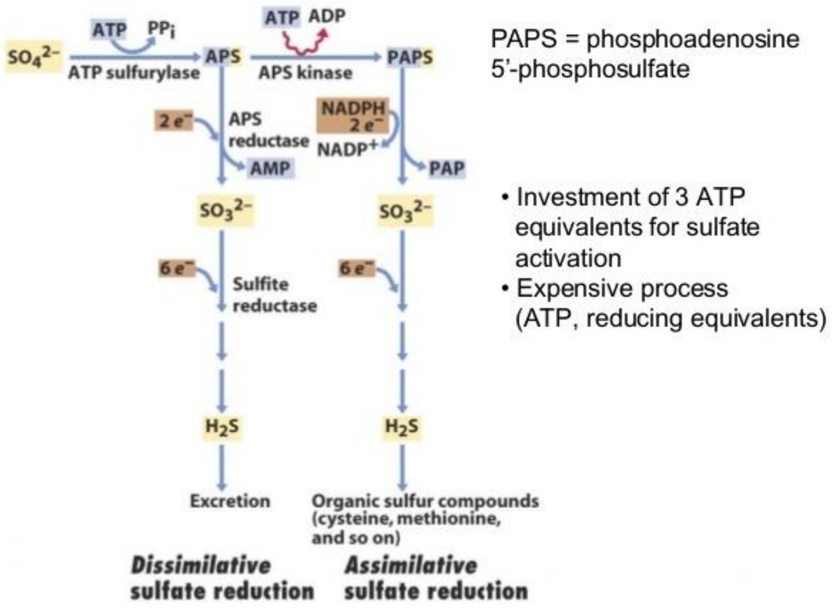


Figure 1-7 – Dissimilatory and assimilatory sulphate reduction (ATP=adenosine triphosphate; ADP=adenosine diphosphate; APS=adenosine 5´-phosphosulphate; PPi=inorganic pyrophosphate; AMP=adenosine monophosphate; PAPS=phosphoadenosine 5´phosphosulphate; PAP=phosphoadenosine 5´-phosphate; NADPH=nicotinamide adenine dinucleotide phosphate hydrogen; NADP$^+$=nicotinamide adenine dinucleotide phosphate (adapted from Brock Biology of Microorganisms 11/e, 2006 Pearson Prentice Hall, Inc).

In the dissimilatory pathway, also referred as sulphate respiration, sulphate is used as an external electron acceptor in the oxidation of energy substrates and sulphide is produced (Bates, 1995; Muyzer and Stams, 2008). SRB are the major group of microorganisms involved in this sulphate respiration.

In assimilatory sulphate reduction, small amounts of sulphate are reduced and consequently sulphide is not usually produced in detectable amounts (Leustek *et al.*, 2000).

The first step of sulphate reduction is the activation of sulphate ion and this step is common in both assimilatory and dissimilatory pathways. ATP promotes this activation by the enzyme ATP-sulfurylase that catalyses the binding of sulphate to the phosphate group of the ATP molecule with the consequent formation of adenosine 5´-phosphosulphate (APS) and inorganic pyrophosphate (PPi). This PPi is hydrolysed by an inorganic pyrophosphatase (pyrophosphate phosphohydrolase) and consequently phosphate is formed (Peck, 1961; Postgate, 1984; Madigan *et al.*, 2003; Barton and Fauque, 2009).

In dissimilatory sulphate reduction (DSR), sulphite (SO_3^{2-}) is directly produced by the APS reduction, catalysed by APS-reductase, and AMP is released.

In assimilatory sulphate reduction, PAPS is formed by the addition of other phosphate group to APS. This reaction occurs in the presence of the enzyme APS-kinase. The PAPS is subsequently reduced to sulphite, by PAPS reductase, releasing of PAP (Peck, 1961; Postgate, 1984; Madigan *et al.*, 2003; Barton and Fauque, 2009).

The reduction of six electrons, in sulphite reduction to sulphide, should compensate the energy consumed in sulphate activation and yield additional ATP for growth (Barton and Fauque, 2009).

The metabolic mechanisms of sulphite reduction has not been not consensual, thus, two mechanisms were proposed (Postgate, 1984; Madigan *et al.*, 2003; Barton and Fauque, 2009):

1) The sulphite reduction to sulphide is catalysed by dissimilatory sulphite reductase (DRS), occurring in one step and no free intermediates are formed.
2) The sulphite reduction to sulphide occurs in three steps and two free intermediates, trithionate ($S_3O_6^{2-}$) and thiosulphate ($S_2O_3^{2-}$), are used.

Actually, Santos and colleagues (2015) recently clarify the enzymology of the sulfate reduction pathway in both bacteria and archaea. The authors proved that the reduction of sulphide is performed by DsrAB, a heterodimer of the phylogenetically related DsrA and DsrB subunits. The protein-based trisulphide is formed by the reduction of the sulfite intermediate which results in the linkage of two cysteine residues of DsrC to a third sulfur atom from sulphite. Thus, the cellular levels of DsrC showed to be a determinant factor in the sulphate reduction rates (Santos *et al.*, 2015).

In assimilatory sulphate reduction the sulphide is immediately converted into organic sulphur compounds, such as amino acids. However, in dissimilatory reduction, the sulphide formed is released to the environment and may dissociate according to the environmental conditions (Madigan *et al.*, 2003). For instance, H_2S can be dissociated to HS^- and H^+ (pH~6.9 and at 25°C) and consequently, under appropriate conditions, HS^- can be then dissociated in S^{2-} and H^+ (pH~12.9 and at 25°C) (Johnson and Hallberg, 2005).

The optimal pH for SRB growth and activity is around 7, thus H_2S and HS^- are the predominant sulphide forms. This biologic hydrogen sulphide can react with some metals dissolved in contaminated waters with the consequent formation of insoluble precipitates (White *et al.* 2003, Costa and Duarte 2005, Vega-López *et al.* 2007). In this process the concentrations of sulphate and dissolved metals are reduced. Therefore, SRB proved to have a high potential in bioremediation processes (da Costa, 2013).

1.4.2. Biological Mechanisms in metals removal

Biologic mechanisms have been explored for removal and/or recovery of metals from aqueous solutions using microorganisms, mostly bacteria. For that purpose, efficient processes for treatment of soils and waters contaminated by metals have been developeded. These mechanisms can be classified in two principal categories: the metabollically passive processes (e.g. biosoption) and metabolically active processes (e.g. bioaccumulation, bioprecipiation, biomineralization, biotransformation) (Tsezos *et al.*, 2012).

Metabollically passive process - Biosorption

Biosorption represents an innovation biotechnological mechanism for recovery of precious metals from aqueous solutions because of its advantages, including low cost, high efficiency, minimal generation of chemical or biological sludge, regenerability, and possible recovery of metals by desorption (Vijayaraghavan and Yun, 2008; Schröfel and Kratošová, 2011).

Biosorption is a metabolic passive process that consists in metals bounded to functional groups of the surface of the cell walls and uses dead biomass. The difference between this process and the conventional adsorption or ion-exchange is the sorbent that in biosorption is biologic (Lovley and Coates, 1997; Chojnacka, 2010). In a biosorption process the dissolved metal ions can be sequester from dilute complex solutions, fast and efficiently (Norton *et al.*, 2004; Park *et al.*, 2010a).

Microorganisms with an abundance of functional groups on their cell surface and extracellular polymers are advantageous to passively sequester metal ions. Thus, various types of biomasses have been used with success in biosorption processes, such as, bacteria, fungi, algae, agricultural and industrial by-products, and other biomaterials (Vijayaraghavan and Yun, 2008). Table 1-3 show different biosorbents used in biosoption of some precious metals (Au(III), Pt(IV) and Pd(II)).

Table 1-3 –Biosorption of Au(III), Pt(IV) and Pd(II) using different biosorbents (adapted from Won *et al.*, 2014)

Metal	***Biosorbent***	**Qmax (mg/g)**	**References**
Au(III)	*Cladosporium cladosporioides*Strain 1	94.2	Pethkar *et al.* (2001)
	C. cladosporioides Strain 2	104.3	Pethkar *et al.* (2001)
	Streptomyces erythraeus	6	Savvaidis (1998)
	Spirulina platensis	5.55	Savvaidis (1998)
	Sulfur derivative of chitosan	630.29	Arrascue *et al.* (2003)
	Glutaraldehyde cross-linked chitosan	571.2	Arrascue *et al.* (2003)
	Dealginated seaweed waste	78.79	Romero-Gonzalez *et al.* (2003)
	Hen eggshell membrane	618	Ishikawa *et al.* (2002)
	Ca-alginate beads	290	Torres *et al.* (2005)
Pt(IV)	*Desulfovibrio desulfuricans*	62.5	de Vargas *et al.* (2004)
	D. fructosivorans	32.3	de Vargas *et al.* (2004)
	D. vulgaris	40.1	de Vargas *et al.* (2004)
	Thiourea derivative of chitosan	386.9	Guibal *et al.* (2000)
	Glutaraldehyde cross-linked chitosan	304.1	Guibal *et al.* (2000)
	Poly(allylamine hydrochloride)-modified *E. coli*	348.8	Mao *et al.* (2010)
	PEI-modified *E. coli*	108.8	Won *et al.* (2010)
Pd(II)	EN-lignin	22.7	Parajuli *et al.* (2008)
	Bayberry tannin immobilized collagen fiber membrane	33.4	Ma *et al.* (2006)
	Racomitrium lanuginosum	37.2	Sari *et al.* (2009)
	l-lysine modified cross-linked chitosan resin	109.5	Fujiwara *et al.* (2007)
	Glycine modified cross-linked chitosan resin	120.4	Ramesh *et al.* (2008)
	Chitosan (glutaraldehyde cross-linked)	180	Ruiz *et al.* (2000)
	Polyallylamine hydrochloride-modified *E. coli* biomass	265.3	Park *et al.* (2010b)
	Chitosan (thiourea derivative)	277.5	Guibal *et al.* (2002)
	Chitosan (glutaraldehyde cross-linked)	287.4	Guibal *et al.* (2002)
	Chitosan (rubeanic acid derivative)	352	Guibal *et al.* (2002)
	PEI-modified *C. glutamicum*biomass	176.8	Won *et al.* (2011)

The biosorption process can be applied in soils, aquifers and groundwater contaminated by metals, for their concentration (Lovley and Coates, 1997).

Metabolically active process – Bioaccumulation and Bioprecipitaion

Bioaccumulation is a metabolic active process performed with living cells (Vijayaraghavan and Yun, 2008; Chojnacka, 2010). The process involves just live cells and the contaminants are transported to the cell and the metals accumulation occurs inside the cell. The bioaccumulation process occurs in two steps, in which the first is faster and similar to biosorption, while the second step is slower and consists in the metal transport into the cells, mostly via energy active transport systems (Aksu and Dönmez, 2000; Chojnacka, 2010). Then, the metal inside the cells can precipitate or be transformed in other species through reactions of oxidation or reduction (Wang and Chen, 2006; Yilmazer and Saracoglu, 2009).

Despite of bioaccumulation process achieved lower residual concentration of metal in solution (Chojnacka, 2010), it is a complex process with associated costs, requiring nutrients for cell maintenance (Vijayaraghavan and Yun, 2008; Chojnacka, 2010).

The biosorbents can limit the efficiency of both biosorption and bioaccumulation processes. The biosorbents should have high metal-binding capacity (Wang and Chen, 2009) and in bioaccumulation processes the cells have to be resistant to high concentrations of metals and not have protection mechanisms for metals accumulation inside the cells (Deng and Wilson, 2001; Kocberber and Dönmez, 2007). Normally, the cells used in this process should have intracellular binding mechanisms that allow the metals binding to the cell. Some of these mechanisms can be the synthesis of low molecular weight proteins as a response to the presence of metal ions in the growth medium (Martin-Gonzalez *et al.*, 2006). For instance, extracellular polymeric substances (EPS) that are usually involved in bioaccumulation processes are produced by some bacteria belonging to genera *Bacillus*, *Acinetobacter*, *Pseudomonas* and to SRB (Beech and Cheung, 1995; Pirog, 1997; Flemming and Wingender, 2001; Guibaud *et al.*, 2005; Ueshima *et al.*, 2008).

Bioreduction can be considered a microbial transformation in which metals or metalloids are transformed by microorganisms through reduction/oxidation or alkylation reactions. Many aerobic and anaerobia bacteria can reduce the metals to a lower redox state (Lovley and Coates, 1997). Wall and Krumholz (2006) report the reduction of U(VI) to U(IV) and Cheung and Gu (2007) report the reduction of Cr(VI) to Cr(III).

The metals reduction can occur as a detoxification mechanism or as a metabolic process in which metals are used as electron acceptors in anaerobic respiration - dissimilatory metal reduction (Lovley 1993; Slobodkin, 2005). SRB, thermophilic microorganisms, Fe(III)-reducing bacteria and fermentative bacteria are some microorganisms that can be responsible for dissimilatory metal reduction (Slobodkin, 2005; Wall and Krumholz, 2006; Cheung and Gu, 2007; Mohapatra *et al.*, 2010).

Bioreduction can also be applied to PGM and studies based on its use have been increased. For instance, the reduction of Pd(II) to Pd(0) on the surface of *Desulfovibrio desulfuricans* with hydrogen as electron donor can be mediated by a hydrogenase (Lloyd *et al.*, 1998) and this process was applied to industrial wastes containing soluble Pd(II), for Pd(0) recovery (Yong *et al.*, 2002b). Other authors reported the use of a mixed consortium of SRB to reduce Rh(III) (Ngwenya and Whiteley, 2006) and Pt(IV) (Rashamuse and Whiteley, 2007) and the PGM removal mechanism has also been subject of study. Riddin and colleagues (2009) demonstrated that two different hydrogenase enzymes are involved in the bioreduction of Pt(IV) into Pt(0), the oxygen-sensitive cytoplasmic hydrogenase promotes the reduction of Pt(IV) to Pt(II) and then this Pd(II) is reduced to Pt(0) by an oxygen tolerant/protected periplasmic hydrogenase.

The microbial mediated precipitation are commonly mentioned as biomineralization or bioprecipitation processes, which involves the microbially induced precipitation of metals and radionuclides as sulphides (Lebranz *et al.*, 2000; White and Gadd, 1998; White and Gadd, 2000), hydroxide (Banfield *et al.*, 2000; Langley and Beveridge, 1999), phosphate (Lebranz *et al.*, 2000) or carbonates (Diels *et al.*, 1999).

One of the most important environmental biotechnological applications in sulphide bioprecipitation is the use of SRB that reduce sulphate to sulphide. However, other bacteria species are also involved in bioprecipitation processes, such as *Citrobacter* sp. that enzymatically produced HPO_4^{2-} that can precipitate metals as phosphates (Brierley, 1990; Macaskie *et al.*, 1992; Macaskie *et al.*, 1994).

1.4.3. Applications of the precious metals biologically recovered

The recovery of Pd(0) from industrial wastes containing soluble Pd(II) (Yong *et al.*, 2002a) and the Pd(0) properties have been reported in several studies. For instance, the biorecovered Pd(0) showed to be highly active catalytically (Mabbett *et al.*, 2006) and the biogenic Pd NPs efficiently catalyse the reduction of chromate (Mabbett *et al.* 2004), the degradation of perchlorate and nitrate (De Windt *et al.* 2006), and the dehalogenation of PCBs (Baxter-Plant *et al.* 2003; De Windt *et al.* 2005), Polybrominated diphenyl ethers (PBDEs) (Harrad and Porter, 2007; Deplanche *et al.* 2009), trichloroethylene (TCE) (Hennebel *et al.*

2009a; Hennebel *et al.* 2009b), chlorophenols (Baxter-Plant *et al.* 2003), lindane and chlorobenzenes (Mertens *et al.* 2007). Thereafter, Pd(0) NPs formation by *C. pasteurianum* BC1 was coupled with the microbial generation of hydrogen to reductively immobilize aqueous chromate using biohydrogen as the electron donor (Chidambaram *et al.* 2010). In 2013, authors reported the preparation of palladium NPs supported upon cells of *Desulfovibrio desulfuricans* (bio-Pd) and their use as a recoverable catalyst for reactions that are a generic model for platform chemical synthesis (Bennett *et al.*, 2013).

The formation of PdS in palladium bioremoval processes was also mentioned and various applications of this metallic sulphide have been suggested. PdS is a semi-conductor (Eg = -2 eV) and has a high potential for several technological applications: in acid resistant high temperature electrodes (Yang *et al.*, 1996), in a wide range of catalytic processes (Birri *et al.*, 2007) and due to its electronic properties, in new potential applications, such as, lithographic films and plates, photographic films, catalytic photoelectrodes and solar cells (Ferrer et al, 2007). However, most of the methods used to prepare PdS are under patent (Dey *et al.*, 2004).

Hence, the recovery of Pd from aqueous solution is interesting independently of the Pd particles formed (Pd(0), Bio-Pd or even of the PdS NPs (or not)) due to the different applications of these particles and only depending of the approach and the final objective.

The application of Pt(0) and Pd(0) bioaccumulated in *D. desulfuricans* cells was also investigated in carbon paper and tested as anode in a polymer electrolyte membrane (PEM) fuel cell for power production (Yong *et al.*, 2007). The same strategy was used with yeast-based biomass, immobilized in polyvinyl alcohol cryogels, for the production of fuel cell Pt(0), which were used to generate electrical energy from renewable sources such as glucose and ethanol (Dimitriadis *et al.* 2007). Platinum also has a wide range of applications, that includes: industry (chemical, chemical/petroleum refining and automotive), jewellery, electronics, medicine (anticancer drugs and dentistry) (Ravindra *et al.*, 2004) and platinum sulphide has been used in the hydrogenation of halogen-containing nitro compounds to amines with high selectivity and on dehalogenation (Dovell and Greenfield, 1965) and reductive alkylation (Dovell and Greenfield, 1967). The platinum can be successfully recovered by biosorption and consequent (Polyethyleneimine) PEI modified biomass incineration (Won *et al.*, 2010). A similar study was carried out using gold solution and biomass of *Sargassum* sp. with high efficiency in gold recovery (Sathishkumar *et al.* 2010).

Gold particles have also a wide range of applications in jewellery, high-tech industries, medical applications properties and electronic and electrical industries (Ramesh *et al.*, 2008; Baba, 1987). In recent years, the use of gold NPs was extended to be used in DNA recognition, hereditary medicine in hyperthermia of tumour, optical coatings, scanning tunnelling microscopes as conductive tips and in catalysis (Cai *et al.*, 2011; Binupriya *et al.*, 2010; Shankar *et al.*, 2005). For instance, the biomass with Au(0) NPs is an efficient cataliser in the reduction of aqueous 4-nitrophenol (4-NP) (Sharma *et al.*, 2007) and some nanogold pharmaceutical applications was already reported, like surface plasmon absorption, surface plasmon light scattering, and biosensing, diagnostic and therapeutic applications of Au NPs (Alanazi *et al.*, 2010). A specific application of Au NPs is for targeted therapy in pancreatic cancer (Patra *et al.*, 2010).

The Ag NPs were also applied to catalyse the reduction of 4-NP (Jia *et al.*, 2008) and can be used for infection protection (wound coatings, bone cements and implants) or prophylactic environment (paints, disinfectants) due to their antibacterial effects (Schröfel and Kratošová, 2011). Silver has regenerative properties (skin regeneration) and wound-healing ability (dressing for burns and ulcers) (Chaloupka *et al.* 2010), as well as, antibacterial, antiviral, antifungal properties (Nair and Laurencin, 2007).

Independently of the different types of nanomaterials (titanium, copper, magnesium or alginate) Au and Ag NPs have promising antibacterial properties and showed good efficiency against bacteria, viruses and fungi (Rai *et al.* 2009). Metallic NPs of silver and gold, presents a broad-spectrum of antimicrobial properties that enhanced their use as disinfectants in purification processes, such as, water and air purification, food production, cosmetics, medicine, clothing, and numerous household products (Marambio-Jones and Hoek 2010).

1.5. Metallic Nanoparticles

In recent years, nanoscience and nanotechnology have attained a huge growth in both research and application fields. The characteristics and properties of nanomaterials during synthesis may be controlled, depending on their diverse applications (Choi *et al.* 2010a, 2012a; Lee *et al.* 2010; Kwon *et al.* 2012). Some of nanotechnological applications are: bioanalysis, diagnostic, therapeutics, medical devices, NPs as tools in medicine and in protein design for optical information processing (Singh *et al.*, 2011).

Nanotechnology defines nanoparticle as small objects that behave as a whole unit in terms of its transport and properties (Singh *et al.*, 2011). NPs are particles where at least one dimension measures less than 100 nm.

The principal parameters in NPs characterization are: shape, size and the morphological sub-structure of the substance. NPs can be presented as aerosols, suspensions or emulsions (Singh *et al.*, 2011).

NPs may be divided in organic and inorganic NPs. The organic NPs include carbon NPs (fullerenes) and some of the inorganic NPs include magnetic NPs, noble metal NPs (gold and silver) and semiconductor NPs (titanium dioxide and zinc oxide) (Singh *et al.*, 2011).

NPs have the potential to fill the gap between atomic or molecular structures and bulk materials. However, these nanosized particles are not always desirable. Some examples are the ferroelectric NPs (<10 nm) that can invert their magnetization direction at room temperatures, rendering them useless for memory usage. Other example is the copper NPs (~50 nm) that exhibit super-hard features, losing the bulk material's characteristic ductility and malleability (Mehta, 2011).

Basically, there are three main approaches for the metallic NP synthesis: physical, chemical and biological. Nevertheless, in a structural approach, two methods can be pointed out: bottom-up and top-down. Bottom-up approach is a process in which first off all the building units are formed and, subsequently, the NPs or nanostructures are formed. The NPs obtained by this approach do not have structural defects and have homogenous chemical compositions. Top-down approach consists in the reduction size of the starting bulk material by mean of mechanical or chemical methods. However, the NP obtained are structurally imperfect, which can influence their physical properties and their surface chemistry behaviour (Schröfel and Kratošová, 2011).

Biosynthesis of NPs can be classified as a bottom-up approach and the principal reaction usually is reduction/oxidation. The biological synthesis respects the green chemistry principles, since the bio-organisms are eco-friendly, as well as the reducing agent employed and the capping agent used in the reaction (Li *et al.*, 2007). In a green chemistry perspective, the three main steps in NPs preparation are:

1) choice of the solvent medium used for the synthesis;

2) choice of an environmentally benign reducing agent;
3) choice of a non-toxic material for the stabilization of the NPs.

The chemical synthesis methods present some disadvantages comparing to biosynthesis. For example, the chemical synthesis of NPs leads to the presence of some toxic chemical species adsorbed on the NPs surface, which may have adverse effects in medical applications (Parashar *et al.*, 2009a, b). On the other hand, biosynthesized NPs are eco-friendly and biocompatible for pharmaceutical applications. Other advantages include the performance at mild temperatures, as at room temperature, with less energy input. Moreover, several metals that have never been chemically synthesized can be biosynthesized (Park *et al.*, 2016).

1.6. Scopes and Organization of this Thesis

The present work aims to explore the potential of liquid-liquid extraction methods, biological methods and a combination of both, to recover precious metals (Au, Ag, Pd and Pt) from aqueous media, preferably as metallic NP. For that purpose and relying on the knowledge already acquired, this thesis includes:

- the use of liquid-liquid extraction strategies, without phosphorus atoms, for the separation of palladium and platinum from aqueous solutions, based on the use of new solvent extraction molecules, diamine derivatives (malonamides and succinamides);
- the investigation of the potential of anaerobic bacteria for the recovery of precious metals (palladium, platinum, gold and silver) and molecular characterization of the bacterial consortia able for biorecovery, as well as the investigation of the mechanisms involved.
- the conjugation of both chemical (SX) and biological methods in order to allow the biorecovery of palladium and platinum, from the loaded solutions resulting from SX. For that purpose previously selected bacterial communities were tested.

Figure 1-8 represents an organizational scheme of the theses.

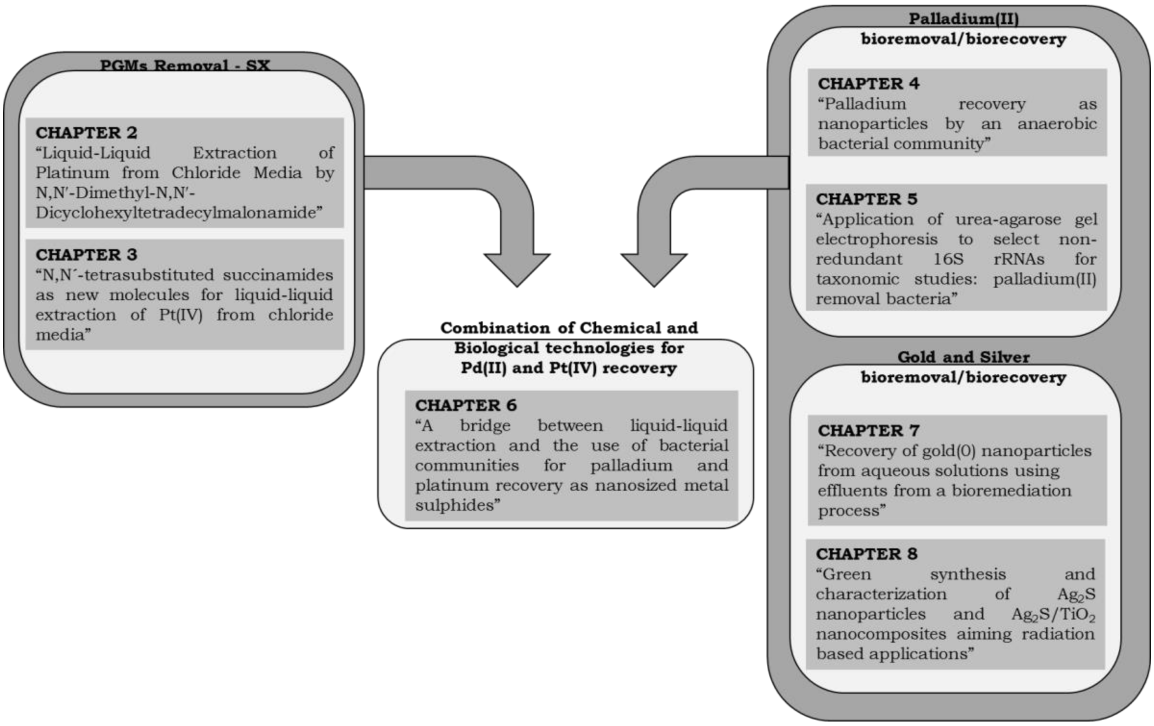


Figure 1-8 – Thesis organization scheme

To achieve the proposed aims, experimental work that resulted in seven scientific papers, was developed.

The present thesis is organized by chapters, each one corresponding to a scientific article, except chapters 1 and 9 which correspond to the General Introduction and Conclusions and Future Perspectives, respectively.

In **Chapter 2**, the potential of a new N,N'-tetrasubstituted malonamide derivative, N,N´-dimethyl-N,N´-dicyclohexyltetradecylmalonamide, (DMDCHTDMA), for Pt(IV) extraction from hydrochloric acid media was demonstrated. In fact, this particular malonamide can be considered a promising extractant for the recovery of platinum (IV) from the typical hydrochloric acid from solutions coming from leaching of Pt-rich sources. N,N'-succinamide derivatives, were also tested, as planned. The use of succinamides instead of malonamides for Pt(IV) extraction was based on the longest distance between the carbonyl groups of these molecules, due to the existence of an additional carbon atom placed between them, which could be theoretically more suitable to "accommodate" the high sized platinum extracted species. Thus, in **Chapter 3**, for the first time, two succinamide derivatives, *N*,*N*′-dicyclohexyl-*N*,*N*′-dimethylsuccinamide (DMDCHSA) and *N*,*N*′-dimethyl-*N*,*N*′-diphenylsuccinamide (DMDPHSA) were synthesised and proved to be also potential extractants for the liquid-liquid extraction of Pt(IV) from hydrochloric acid solutions. **Chapter 4** reports the biorecovery of Pd(II) as NPs by a bacterial consortium, demonstrating the biotechnological potentialities of this community. Thus, aiming to identify the bacteria involved in Pd(II) removal, the separation of urea/heat-denatured DNA fragments by urea–agarose gel electrophoresis, was applied, for the first time, to select 16S rRNA-cloned amplicons for taxonomic studies (**Chapter 5**).

The removal of platinum and palladium from aqueous solutions was successfully performed using chemical and biological methods, respectively. However, in SX experiments the platinum was not recovered in a solid phase. Therefore, in **Chapter 6**, the potentialities of combining liquid-liquid extraction and the use of bacterial communities for the recovery of palladium and platinum as nanosized metal sulphides, were demonstrated.

The biorecovery of gold from aqueous solutions, as metallic gold NPs, using the biogenic sulphide present in an effluent from an AMD bioremediation process was also demonstrated. This process, described in **Chapter 7,** allows the treatment of sulphide in an effluent and the simultaneous achievement of Au(0) NPs with high economic value. This work resulted in a scientific paper and led also to the request of an international patent. The same effluent was efficiently used for the synthesis of Ag_2S, both in batch and in a continuous system, and also for the synthesis of Ag_2S/TiO_2 nanocomposite. The Ag_2S NPs and the respective TiO_2 nanocomposite showed to have semiconductor properties that might confer them several applications, namely as photocatalysts and as additives in low-cost photovoltaic cells (**Chapter 8**).

Considering the increasing need of precious metals recovery (Pt, Pd, Au and Ag) from secondary sources, I believe that the results presented in this work are promising and open the possibility of combining several strategies for the development of efficient and viable processes aiming the recovery of precious metals from secondary sources.

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CHAPTER 2

Liquid-Liquid Extraction of Platinum from Chloride Media by N,N′-Dimethyl-N,N′-Dicyclohexyltetradecylmalonamide

ABSTRACT

Liquid-liquid extraction of platinum (IV) from chloride media was carried out using *N,N'*-dimethyl-*N,N'*-dicyclohexyltetradecylmalonamide (DMDCHTDMA) in 1,2-dichloroethane. Platinum can be effectively extracted by DMDCHTDMA without addition of tin(II) chloride, since extraction percentages (%E) of 88% and 99% have been achieved from 6 M and 8 M HCl, respectively. Moreover, platinum can be successfully stripped through a simple contact with a 1 M HCl solution. The effect of some experimental parameters such as equilibration time, diluent, extractant and HCl concentrations was systematically investigated. The loading capacity of DMDCHTDMA was also evaluated. Data obtained from successive extraction-stripping cycles suggest a good stability pattern of DMDCHTDMA. Preliminary extraction data achieved with single metal ion solutions pointed out to a possible separation of platinum (IV) from palladium(II).

Keywords: Platinum, malonamide derivatives, liquid-liquid extraction, hydrochloric acid

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1. INTRODUCTION

Platinum is a widely used metal nowadays; its multiple applications have radically increased in the last century, spreading from catalysis, jewelry, electronic and electric devices, to medical instruments, dental equipment, space materials, and even included in anticancer drugs. One of its essential uses is in automotive catalytic converters, where it converts the harmful emissions into carbon dioxide, nitrogen, and water. Nearly one-third of newly mined platinum is estimated to be used in this fashion. In addition, new applications are being discovered almost daily, amidst a supply that is extremely scarce (Wołowicz and Hubicki, 2009). In fact, platinum is a very rare metal in the Earth's crust, the worldwide reserves being concentrated only in two regions, South Africa and Siberia (Marinho *et al.*, 2011).

As a result of its high value, rising demand and the progressive depletion of the primary raw material resources, there is a growing interest on the recovery and reutilization of platinum from industrial wastes, including automotive spent catalysts, catalysts and electronic devices, as well as of other platinum group metals (PGM) (Wołowicz and Hubicki, 2009). Most ordinary recovery methods reported in the literature for PGM recovery from catalytic converters are based on the full dissolution of the waste sample in an oxidative medium (Marinho *et al.*, 2011; Angelidis and Skouraki, 1996; Marinho *et al.*, 2010; Atkinson *et al.*, 1992; Barakat and Mahmoud 2004). Thus, the development of processes aiming at the efficient separation and recovery of PGM from highly acidic pregnant leaching solutions is desirable.

The application of liquid-liquid extraction to PGM recovery is not a new subject; accordingly, a relevant work collecting some older but relevant information about the main extractants explored to date, and of the chemical behavior of PGM chlorocomplexes as well, deserves a mention (Al-Bazi and Chow, 1984). Concerning the increasing need for recovery of PGM from secondary sources such as the automotive catalysts, liquid-liquid extraction strategies have again been considered a promising alternative since the 1990s for this specific purpose (Marinho *et al.*, 2010), and thus several extractants for platinum, recovered from spent samples or included in standard chloride solutions, have been extensively investigated in the last years. Those extractants fall mainly into two groups, amines and organophosphorus compounds; although others such as dialkylsulphoxides (Preston and Preez, 2002) have also been reported. In the first group, compounds such as tri-isooctylamine (Alamine 308) (Lee *et al.*, 2009a, 2009b), tri-octyl/decylamine (Alamine 336) (Barakat and Mahmoud, 2004; Sanuki *et al.*, 1999) and mixtures of trioctylamine and tridecylamine (Nowottny *et al.*, 1997) and ammonium quaternary salts such as Aliquat 336 (Marinho *et al.*, 2010; Lee *et al.* 2009a), have been reported, amines being of particular interest because platinum can be extracted without the interference of metals such as iron and aluminium (Lee *et al.*, 2009b). In the second group, tributyl phosphate (TBP) (Sanuki *et al.*, 1999), 2-ethylhexyl phosphonic acid mono-2-ethylhexyl ester (PC-88A) (Bandekar and Dhadke, 1998), tri-octyl phosphine oxide (Cyanex 921) (Mhaske and Dhadke, 2001; Benguerel *et al.*, 1994) and bis(2,2,4-trimethylpentyl)monothiophosphinic acid (Cyanex 302) (Kumar *et al.*, 2008) are relevant examples, but these organophosphorus extractants are not so environmentally friendly due to the presence of the phosphorus atoms.

Recently, a few amide derivatives have been proposed for the solvent extraction of PGM from hydrochloric acid solutions (Narita *et al.*, 2005; Narita *et al.*, 2008; Belair at al., 2011) and amongst them, N,N-tetrasubstituted malonamides have been investigated. In previous publications, the potential of *N,N'*-dimethyl-*N,N'*-diphenyltetradecylmalonamide (DMDPHTDMA) for the extraction of platinum (Malik and Paiva, 2009), rhodium (Malik and Paiva, 2008), and later on its capacity to separate palladium, platinum, and rhodium (Malik and Paiva, 2010) has been emphasized. Moreover, the extraction capability shown by *N,N'*-dimethyl-*N,N'*-dicyclohexylmalonamide (DMDCHMA) towards ruthenium has also been investigated (Malik and Paiva 2011). The results of platinum extraction from hydrochloric acid media using DMDPHTDMA showed that, to achieve an efficient metal extraction, the use of a labilizing agent such as tin(II) chloride is necessary (Malik and Paiva, 2009).

The aim of the present work is to reveal some new information about the use of a different N,N-tetrasubstituted malonamide derivative, DMDCHTDMA, to recover platinum (IV) from hydrochloric acid media, envisaging the finding of a suitable platinum extractant to be applied on the treatment of specific industrial leaching or effluent solutions containing this rare metal, namely those coming from the processing of secondary sources.

2. EXPERIMENTAL

2.1. Synthesis

Tetrabromodecane (≥97%) from Fluka, sodium hydride (60% in mineral oil) from Aldrich and toluene (99.8%, HPLC grade) from LAB-SCAN, were used without further purification.

FTIR spectrum was recorded in a Bruker (Tensor 27) spectrophotometer, in a KBr pellet. TLC analysis was performed in Merck silica plates, which were eluted with ethyl acetate and revealed with iodine. The melting point was measured on a SRS EZ-Melt Automated melting point apparatus. 1H spectrum was acquired on a Bruker Avance 400, 400 MHz spectrometer, using $CDCl_3$ as solvent. The LC–MS system was an Agilent Technologies 1200 Series LC coupled to a Bruker Daltonics HCT ultra (ion trap detector). The ionization was made by electrospray (ESI) in the positive polarity. Typical ESI conditions were: nebulizer pressure 50 psi, capillary voltage 4.0 kV, and drying gas temperature 300°C. A GraceSmart column was used (100 mm length, 2.1 mm internal diameter, C18, 3 mm), stabilized at 25°C. The composition of the mobile phase was 10% water / 90% MeOH, both with 0.1% formic acid, and the flow rate was 0.4 mL/min. The fragmentation spectra (MS^2) were obtained using Auto MS(n).

N,N'-Dimethyl-*N,N'*-dicyclohexylmalonamide (DMDCHMA) was obtained as reported previously (Chan *et al.*, 1997; Costa *et al.*, 2007). DMDCHTDMA was synthesized from the latter also by a described method (Chan *et al.*, 1997): DMDCHMA (9 g, 0.031 mol) was dissolved in 25 mL of toluene, in a round bottom flask. Sodium hydride (2.16 g, 0.054 mol) was added under stirring, and the mixture was refluxed for 2 h. The reaction mixture was allowed to cool down to room temperature and a solution of bromotetradecane (8.5 mL, 8.49 g, 0.031 mol) in 20 mL of toluene was added dropwise. The mixture was then refluxed for 48 h, the reaction being followed by TLC. The suspension was filtered over celite and under vacuum. The flask and solid residue were washed with ethyl acetate. The organic solvents were removed in a rotary evaporator, affording a white solid, which was dried in a vacuum oven at 30°C overnight. The yield was 13.7 g (90%) of product, with melting point 47–49°C (lit.(Chan *et al.*, 1997): 53–54°C). FTIR (KBr): 1648, 1630 cm^{-1} (C = O). 1H NMR ($CDCl_3$): 4.436 (1 H, broad singlet, CH cyclohexyl), 3.800 (2 H, 2 × broad singlet, CH cyclohexyl + OC-CH-CO), 2.936 (3 H, broad singlet, N-CH_3), 2.822 (3 H, doublet, N-CH_3), 1.855 (5 H, multiplet, CH_2 cyclohexyl), 1.665 (5 H, multiplet, CH_2 cyclohexyl), 1.386, 1.260 (34 H, multiplet + singlet, CH_2 cyclohexyl + $CH_3(CH_2)_{13}$), 1.092 (2 H, multiplet,

CH_2 cyclohexyl), 0.897 (3 H, triplet, $CH_3(CH_2)_{13}$). LC-MS: Retention time = 4.2 min; m/z = 491 ($[M+H]^+$), 513 ($[M+Na]^+$); MS/MS of the parent ion (491): m/z = 409 ($[M-C_6H_{11}+2.H]^+$, 49%), 378 ($[M-C_6H_{11}NCH_3]^+$, 100%), 327 ($[M-2.C_6H_{11}+3.H]^+$, 36%), 296 ($[M-C_{14}H_{29}+3.H]^+$, 35%), 140 ($[C_6H_{11}NCH_3CO]^+$, 54%).

2.2. Solvent Extraction Experiments

All reagents and solvents were used without further purification. 5×10^{-4} M Pt(IV) solutions were prepared from the $PtCl_4$ salt 98% (Aldrich), which was previously dissolved in the required volumes of hydrochloric acid. 1 M and 6 M HNO_3, and H_2SO_4 solutions, containing similar concentrations of Pt(IV), were prepared in the same way, by dissolution of the Pt(IV) chloride salt in each acid, to investigate platinum extraction from other media than the chloride one.

The organic phases containing 0.05 M DMDCHTDMA, or other desired concentrations, were generally prepared in 1,2-dichloroethane (1,2-DCE, Fluka, minimum 99.5%), although other diluents were also used when the diluent effect was to be investigated: toluene (Panreac, minimum 99.5%) (analytical reagent, Lab-Scan, minimum 99%), kerosene (Sigma-Aldrich, boiling point 175–325 C), dichloromethane (Riedel-de Haën, analytical reagent, minimum 99.8%), Escaid 100® (Exxon Mobil, boiling point 195–245°C, 18–24% aromatic content), Escaid 110® (Exxon Mobil, boiling point 209–228°C, ~1% aromatics), and Shellsol D70® (Shell Chemicals, boiling point 198–242°C, low aromatic content). The addition of 20% (v/v) of 1-octanol to the diluents, other than 1,2-DCE, was also attempted, as well as 1-octanol alone.

Hydrochloric acid solutions containing 9×10^{-4} M Pd(II) were also prepared from the Pd(II) chloride salt 99% (Aldrich), and contacted with 0.05 M solutions of DMDCHTDMA in 1,2-DCE.

Distilled water and 1 M hydrochloric acid solutions were used as agents to strip platinum from the loaded organic phases.

General extraction and stripping experiments were carried out at room temperature by contacting equal volumes of the organic and aqueous phases (A/O = 1) for 30 minutes, by mechanical shaking, adopting a rotation speed between 900 and 1000 rpm. After separation of the two phases, the aqueous phases were filtrated and the platinum contents in the aqueous solutions were determined by UV/vis spectroscopy (Hach Lange, DR 2800) using the tin(II) chloride method at 403 nm (Ayres and Meyer, 1951), or by atomic absorption spectrometry (Atomic Absorption Spectroscopy (AAS), Varian Spectra AA-20) (concept of the technique in Appendix 3), whenever interferences or any difficulties arose with the spectrophotometric method. The procedure for the spectrophotometric determination of platinum has been previously described (Malik and Paiva, 2009; Ayres and Meyer, 1951). For the analysis of palladium, an AAS (Perkin Elmer, AAnalyst 800) with graphite furnace THGAA (transverse) and correction of Zeeman was utilized. Two replicate experiments were carried out for each condition and the analysis of the solutions before and after extraction was performed in triplicate. The coefficient of variance estimated for the results achieved from the different extraction and stripping experiments is approximately ± 5%.

3. RESULTS AND DISCUSSION

3.1. Effect of Hydrochloric Acid Concentration on the Extraction of Platinum (IV)

These experiments were performed using 0.05 M DMDCHTDMA in 1,2-DCE as organic phase and varying the hydrochloric acid concentration in the aqueous media between 1 to 8 M (See Figure 2-1). It can be observed that the efficiency of platinum (IV) extraction (%E) increases as the hydrochloric acid concentration in the aqueous phases also increases. The %E Pt(IV) sharply boosts from 40% to 70% between 4 and 5 M HCl, reaching %E values of nearly 90% and 99% for 6 M and 8 M, respectively.

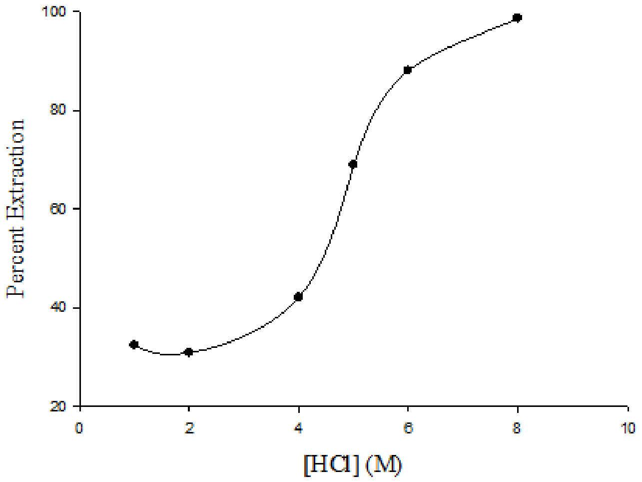

Figure 2-1 - Effect of HCl concentration on Pt(IV) extraction by a 0.05M DMDCHTDMA solution in 1,2-DCE. [Pt] = 5 x 10^{-4} M.

DMDCHMA (*N,N'*-dimethyl-*N,N'*-dicyclohexylmalonamide), DMDPHMA (*N,N'*-dimethyl-*N,N'*-diphenylmalonamide) and DMDPHTDMA (*N,N'*-dimethyl-*N,N'*-diphenyltetradecylmalonamide) were also tested for the extraction of platinum (IV) under a similar set of experimental conditions (Malik and Paiva, 2009). Those three malonamide derivatives do not efficiently extract platinum (IV). The maximum %E was obtained for DMDCHMA at 8 M HCl, and its value was below 30%. This is a similar behavior to that observed for DMDPHTDMA, for which a maximum Pt(IV) extraction, close to 30%, was obtained at 5 M HCl (Malik and Paiva, 2009). For DMDPHMA, no liquid-liquid extraction of platinum was achieved, although the metal ion was not detected in the aqueous phases after contact with the organic media. This occurrence took place due to the formation of a yellow precipitate that appeared in the filters after phase separation. Those precipitates were analyzed by X-Ray Diffraction (XRD) (concept of the technique in Appendixe 3), but their composition could not be determined due to their amorphous nature. However, elemental analysis of the solids collected by Energy-Dispersive X-ray Fluorescence showed very intense platinum emission peaks, confirming that it was a precipitate containing platinum. According to the literature (Malik and Paiva, 2009; Vest *et al.*, 1991; Saito *et al.*, 1989) the presence of some compounds in the aqueous phase such as $SnCl_2$ or $SnBr_2$, or the SCN^- ion, usually helps in platinum extraction.

In fact, Malik and Paiva (2009) concluded that DMDPHTDMA is able to efficiently extract platinum in a single equilibration (%E > 90% for 6 M ≤ HCl ≤10 M), if tin(II) chloride is added to the aqueous phases.

The efficient extraction of platinum (IV) from 6 M and 8 M HCl solutions by DMDCHTDMA without the need of tin(II) chloride, contrarily to DMDPHTDMA, may be an important advantage in view of an eventual industrial utilization of this extractant for platinum separation from hydrochloric acid solutions. In fact, besides the additional costs associated with the use of those salts, tin is extracted together with platinum to the organic phase (Malik and Paiva, 2009), thus increasing the complexity of the extraction system.

By comparison of the results involving DMDCHTDMA with the data mentioned above for other malonamide derivatives (DMDCHMA, DMDPHMA and DMDPHTDMA), it can also be concluded that the structure of the malonamide derivatives (the change of phenyl by cyclohexyl rings, together with the alkylation of the central carbon atom of the malonamide), determines their efficiency towards platinum (IV) extraction from HCl media.

3.2. Extraction of Platinum (IV) from Aqueous Media Other than Chloride

The extraction of 5 × 10^{-4} M platinum (IV) from aqueous media other than chloride was also tested, to better check the role of chloride ions in the extraction process. A 37% extraction of platinum was achieved from nitric acid medium (1 M HNO_3), while 18% were obtained from sulfuric acid medium (1 M H_2SO_4). From 6 M HNO_3, the %E Pt(IV) was lower than 5%. The formation of a third phase occurred when 6M H_2SO_4 was used. These results suggest that chloride ions are determinant for the efficient extraction of platinum (IV). These results reinforce the crucial importance of the platinum chlorocomplexes existence in the aqueous solutions, for which N,N´-tetrasubstituted malonamides show a special affinity (Malik and Paiva, 2009).

3.3. Effect of Equilibration Time on the Extraction of Platinum (IV)

This set of experiments was carried out with 6 M HCl solutions, keeping a 5 × 10^{-4} M platinum (IV) concentration, and using a 0.05 M DMDCHTDMA in 1,2-DCE as organic medium. The time of contact for the two phases varied between 1 and 60 minutes. It was found that the extraction kinetics for this system is extremely favorable. (See Figure 2-2). A contact time of 1 minute results in a 93% Pt(IV) extraction, equivalent to the ones achieved for longer periods of time. It was decided to adopt a contact time of 30 minutes for all the experiments, much longer than that required for the thermodynamic equilibrium of the extraction system to be achieved.

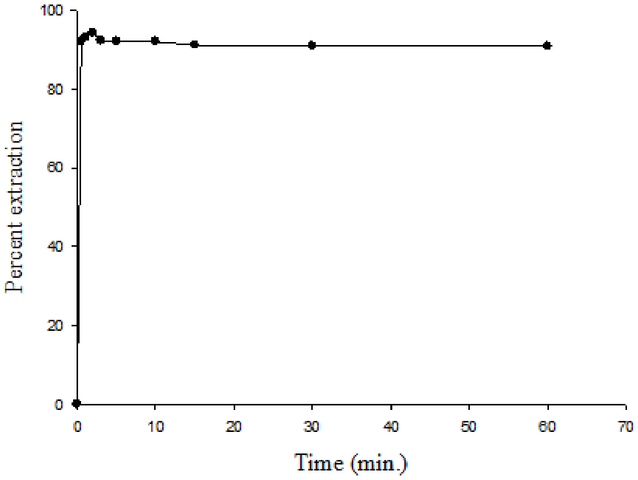

Figure 2-2 - Effect of contact time on Pt(IV) extraction from 6 M HCl by 0.05M DMDCHTDMA in 1,2-DCE. [Pt] = 5 x 10^{-4} M.

3.4. Effect of Different Diluents on the Extraction of Platinum (IV)

Several diluents (commercial and other polar diluents in alternative to 1,2-DCE) were tested, in order to determine their influence on the extraction performance of DMDCHTDMA for platinum (IV). Commercial diluents were mainly selected in view of an industrial application, while 1-octanol was chosen as a polar substance, more environmental-friendly than chlorinated diluents. The extraction experiments were carried out from 6 M HCl and the extractant concentration was kept constant at 0.05 M. DMDCHTDMA was completely soluble in all the mentioned diluents. The overall results obtained are displayed in Table 2-1.

Table 2-1 - Effect of different diluents on the extraction percentage of 5×10^{-4} M Pt(IV) in 6 M HCl by 0.05 M DMDCHTDMA (standard deviation: ± 5%)

Conditions	**%E Pt(IV)**
1,2-Dichloroethane (1,2-DCE) alone	0
1,2-DCE	90
Dichloromethane	66
1-Octanol	100
Toluene	6
Xylene (mixture of isomers)	5
Escaid 100®	0
Escaid 110®	0
Shellsol D70®	0
Kerosene	0
70 % Toluene + 30 % 1,2-DCE	36
70 % Xylene + 30 % 1,2-DCE	39
70 % Escaid 100® + 30 % 1,2-DCE	72
70 % Escaid 110® + 30 % 1,2-DCE	52
70 % Shellsol D70® + 30 % 1,2-DCE	50
70 % Kerosene + 30 % 1,2-DCE	59
80 % Toluene + 20 % 1-octanol	8
80 % Xylene + 20 % 1-octanol	1
80 % Escaid 100® + 20 % 1-octanol	0
80 % Escaid 110® + 20 % 1-octanol	6
80 % Shellsol D70® + 20 % 1-octanol	7

Polar diluents like dichloromethane, and particularly 1,2-DCE and 1-octanol, allow good %E for platinum (IV). 1,2-DCE alone does not extract platinum, confirming the determinant role of DMDCHTDMA on the extraction of the metal ion. Aromatic diluents such as toluene and xylene, and commercial diluents like kerosene, Escaid 100®, Escaid 110®, and Shellsol D70® are not efficient for platinum (IV) extraction with DMDCHTDMA. However, when those diluents are mixed with 30% 1,2-DCE (v/v), the %E Pt(IV) increases significantly, which emphasizes the role of the chlorinated diluents for platinum extraction.

Volume ratios of 1,2-DCE to commercial diluents higher than 30% may eventually allow higher %E Pt(IV) than those depicted in Table 2-1.

In contrast with results seen in the presence of 1,2-DCE in the solvent mixture, 20% of 1-octanol did not improve the %E Pt(IV). However, the results show that the use of 1-octanol alone as diluent for DMDCHTDMA is very successful, since 100% Pt(IV) extraction from 6 M HCl was achieved. This demonstrates that 1-octanol can work as a good alternative to 1,2-DCE, and that the use of polar diluents seems to be relevant to maintain the DMDCHTDMA extraction performance. According to the literature, whereas the usual hydrocarbon diluents promote aggregation of the malonamide extractants (Berthon *et al.*, 2010), polar diluents do not seem to favor this phenomenon; this is possibly the justification of the overall results obtained in this section, as aggregation may be inhibiting Pt(IV) extraction by DMDCHTDMA when commercial diluents are used.

3.5. Extraction Isotherm of Platinum (IV) by DMDCHTDMA

In order to determine the loading capacity of DMDCHTDMA for platinum (IV), adequate experiments were carried out using 2.6×10^{-3} M Pt(IV) in 6 M HCl and 0.05 M DMDCHTDMA in 1,2-DCE solutions. The organic phase was contacted with fresh aqueous solutions using different A/O volume ratios. (See Figure 2-3).

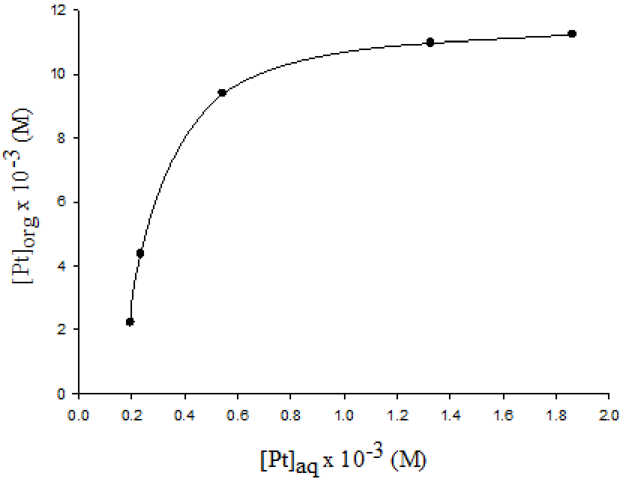

Figure 2-3 - Extraction equilibrium isotherm for Pt(IV) by 0.05M DMDCHTDMA in 1,2-DCE. [Pt] = 2.6×10^{-3} M in 6 M HCl

Under the present experimental conditions, the calculations based on mass balance for the fully loaded organic phase show that the maximum loading of DMDCHTDMA for platinum (IV) is of about 1.1×10^{-2} M Pt, the extractant:Pt molar ratio being 4.5, which can be considered a quite good loading capacity. In fact, comparing with DMDPHTDMA, which in the presence of $SnCl_2$ (bearing a 6:1 Sn:Pt molar ratio) exhibited a loading capacity of 7.19×10^{-4} M Pt (Malik and Paiva, 2009), DMDCHTDMA shows a much higher loading capacity than DMDPHTDMA (about 15 times more).

3.6. Effect of DMDCHTDMA Concentration on Platinum (IV) Extraction

This set of experiments was carried out with 5×10^{-4} M Pt(IV) in 6 M HCl aqueous solutions. DMDCHTDMA concentrations in 1,2-DCE were varied between 0.001 M and 0.1 M.

The %E Pt(IV) increases when higher extractant concentrations are used, but for 0.05 M or higher concentrated DMDCHTDMA solutions no significant differences on %E Pt(IV) were observed. The log-log plot between platinum (IV) distribution coefficients (*D*) and DMDCHTDMA concentrations indicates a slope value near to 1.5, as can be seen in Figure 2-4. Hence, this result suggests that species bearing 1:1 and/or 1:2 metal:extractant stoichiometries should exist in the organic solutions. The slope value obtained may be due to variations of the extractant:platinum (IV) stoichiometries in the DMDCHTDMA concentration range adopted.

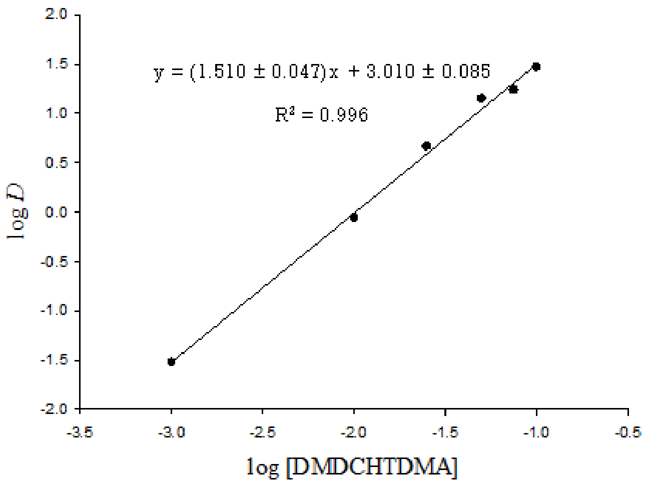

Figure 2-4 - Effect of DMDCHTDMA concentration on Pt(IV) extraction from 6 M HCl. [Pt] = 5 x 10^{-4} M.

Data collected on the extractive behavior of different amine and amide derivatives towards platinum (IV) always point out to the formation of ion pairs: the protonation of the extractant occurs and then its association with the predominant anionic complex of Pt(IV) in concentrated HCl media, $[PtCl_6]^{2-}$, takes place (Yoshizawa *et al.*, 1997; Jyothi *et al.*, 2009; Narita *et al.*, 2006). Accordingly, and taking into account the similarity of the %E Pt(IV) curve *vs.* HCl concentration in the aqueous phase obtained for some diglycolamide and thiodiglycolamide derivatives already investigated (Narita *et al.*, 2006), the occurrence of a similar ion–association pathway involving one or two protonated DMDCHTDMA molecules and $[PtCl_6]^{2-}$ is likely, leading to the formation of neutral adduct species in the organic phase. Although more research is needed to confirm this extraction reaction proposal, this assumption is also supported by the fast extraction rate of Pt(IV) by DMDCHTDMA, in addition to the strong basic character of the extractant (Spjuth *et al.*, 2000).

3.7. Palladium (II) Extraction by DMDCHTDMA

To assess the possibility of using DMDCHTDMA to separate platinum (IV) from palladium (II), some experiments were carried out to evaluate the behavior of the extractant towards Pd(II) in a solution containing this latter metal only. Hence, aqueous phases containing 9 x 10^{-4} M Pd(II) in 1 M to 8 M HCl were contacted with 0.05 M DMDCHTDMA in 1,2-DCE organic solutions, the adopted experimental conditions being similar as before. The %E Pd(II) from 1 and 2 M HCl is below 10%, from 4 M to 6 M HCl is null and at 8 M HCl is around 40%. These results suggest that it may be possible to extract Pt(IV) by DMDCHTDMA and leave Pd(II) in the aqueous phase at 6 M HCl, but further extraction experiments carried out with the simultaneous presence of Pt(IV) and Pd(II) in the aqueous phase are needed to confirm such assumption.

3.8. Stripping of Platinum from the Loaded Organic Phases and Extractant Reutilization

The good %E Pt(IV) obtained was the main reason motivating the search for adequate aqueous solutions to strip the metal ion to a new aqueous phase. For that purpose, distilled water and 1 M HCl were used as stripping agents for platinum (IV). The organic phase (0.05 M DMDCHTDMA in 1,2-DCE) was previously contacted with a 6 M HCl aqueous solution containing 5×10^{-4} MPt(IV) at A/O=1. This as-prepared organic phase, containing around 4×10^{-4} MPt(IV), was therefore contacted with the two stripping agents, again for 30 minutes and A/O =1. It should be pointed out that for the experiments involving the two stripping agents the phase separation is fast and there are no third-phase problems.

The stripping percentages (%S) obtained with distilled water and the 1 M HCl solution were 60% and 90%, respectively. Thus, it can be concluded that Pt(IV) can be easily and quantitatively stripped from the loaded DMDCHTDMA organic phases simply using a 1 M HCl solution.

Among a variety of solutions tested as stripping agents for platinum from loaded DMDPHTDMA, only a mixture of 4 M HCl and 0.05M $NaClO_3$ was able to strip a high percentage of platinum, of about 76%, due to the need of Sn(II) addition to the initial feed aqueous phase (Malik and Paiva, 2009). The advantage of having a N,N´-tetrasubstituted malonamide derivative that efficiently extracts Pt(IV) in the absence of Sn(II), is also extended to the metal ion stripping, since the use of a simpler and diluted HCl solution proved to be enough for Pt(IV) quantitative stripping.

In order to collect information on the stability of DMDCHTDMA for the experimental conditions adopted, five successive extraction-stripping stages were carried out, using fresh aqueous solutions containing 5×10^{-4} M Pt(IV) in

6 M HCl. The results obtained for Pt(IV) in each extraction step are depicted in Figure 2-5.

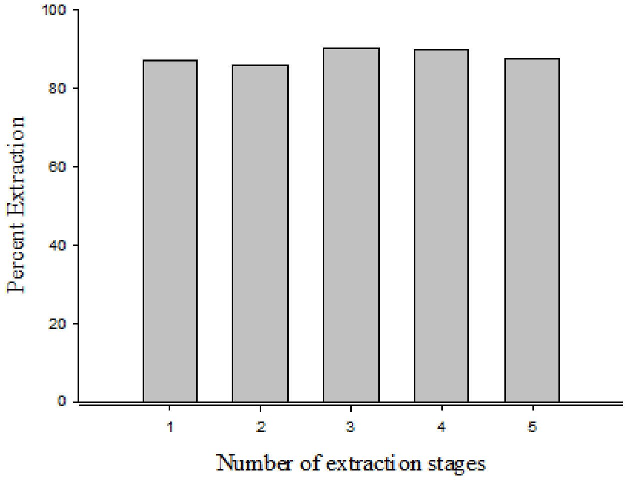

Figure 2-5 - Dependence of the DMDCHTDMA extraction behavior on the number of extraction cycles. [Pt] = 5 x 10^{-4} M in 6 M HCl, [DMDCHTDMA] = 0.05M in 1,2-DCE.

The data achieved clearly denote that the reutilization of DMDCHTDMA is feasible, since no decrease of Pt(IV) extraction is observed after those successive contacts. In addition to the previously investigated characteristics presented by this extractant, the possibility of its reutilization after several extraction and stripping cycles is an important feature envisaging a potential industrial application.

4. CONCLUSIONS

This article points out a new solvent extraction application for a specific *N,N'*-tetrasubstituted malonamide derivative, *N,N´*-dimethyl-*N,N´*-dicyclohexyltetradecylmalonamide, (DMDCHTDMA). In fact, this particular malonamide can be considered a promising extractant for the recovery of platinum (IV) from hydrochloric acid media, since it is able to extract more than 88% Pt(IV) from 6 to 8 M hydrochloric acid solutions, when dissolved in 1,2-dichoroethane, without the need of tin(II) addition. Platinum can be easily and quantitatively stripped to a new aqueous phase by simple contact with a 1 M

HCl solution. The extraction process is kinetically fast and DMDCHTDMA shows quite a good loading capacity towards platinum (IV). Moreover, this extractant can be reutilized several times without losing its extraction ability. 1-Octanol, when used as a diluent for DMDCHTDMA, does not lower the extraction performance of the solvent towards platinum, as occurred when other diluents were tested.

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CHAPTER 3

N,N´-tetrasubstituted succinamides as new molecules for liquid-liquid extraction of Pt(IV) from chloride media

ABSTRACT

Due to the increasing demand of platinum group metals (PGM), among which platinum, the search for new alternatives aiming to their extraction and recovery is nowadays of great importance. Thus, two *N*,*N*′-tetrasubstituted succinamides, *N*,*N*′-dicyclohexyl-*N*,*N*′-dimethylsuccinamide (DMDCHSA) and *N*,*N*′-dimethyl-*N*,*N*′-diphenylsuccinamide (DMDPHSA) were synthesized and investigated, for the first time, as potential molecules for the liquid–liquid extraction of Pt(IV) from chloride solutions. According to the extraction and stripping results obtained in a single contact, these succinamides can be considered promising extractants for the recovery of platinum (IV) from hydrochloric acid media, since DMDCHSA is able to extract more than 95% Pt(IV) from 4 M HCl or higher concentrations, and DMDPHSA extracts 80% and 92% Pt(IV) from 6 M and 8 M HCl solutions, respectively. Furthermore, platinum (IV) can successfully be stripped through a simple contact with seawater: 93% Pt(IV) were recovered from DMDCHSA, while 60% stripping were obtained from DMDPHSA. For each compound, the time necessary to reach equilibrium, and the effects of the type of diluent and different extractant concentrations on the Pt(IV) extraction percentage were investigated. The loading capacity of DMDCHSA and DMDPHSA toward Pt(IV) was also evaluated, and the results show that both extractants present a very high loading capacity. In addition, data obtained from successive extraction-stripping cycles suggest a good stability pattern for both succinamides.

Keywords: Platinum, Succinamide derivatives, Liquid-liquid extraction, Hydrochloric acid.

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1. INTRODUCTION

Platinum is an extremely rare metal, occurring at a concentration of only 0.005 ppm in the earth's crust (Murata, 1958), the worldwide reserves being concentrated in only two regions, Siberia and South Africa (Marinho *et al.*, 2010).

Platinum and the other platinum group metals (PGM) are widely used in many fields, such as catalysts in many chemical processes, in the electrical and electronic industries, as corrosion resistant materials, and in jewelry (Peng *et al.*, 2009). Their excellent selectivity and activity toward reactants, and resistance to oxidation at high temperatures, make them very effective catalysts. For instance, platinum is a common active phase for petroleum cracking, dehydrogenation, reforming, and hydrogenation, all these processes being extensively used in the refining and petrochemical industries. An important use of platinum is still in the composition of the catalytic converters, which are part of the exhaust systems in automobiles (although the replacement of platinum by palladium is the most recent tendency). Automotive catalysts are determinant in reducing the emission of the harmful carbon monoxide, light hydrocarbons and nitric oxide into the atmosphere, converting them into carbon dioxide, nitrogen and water. About half of the newly mined platinum is used for this purpose.

Worldwide efforts are being made to recover and refine PGM from various spent materials (Barakat and Mahmoud, 2004). Furthermore, the potential environmental hazards of spent industrial and automotive catalysts justifies the development of recovery and recycling policies for these waste materials.

There are many methods for the recovery of platinum and other PGM from spent catalysts, including from automobile catalytic converters. However, the fraction recovered from such secondary sources reaches only about 40% of the overall amount (Yang, 2009). When hydrometallurgical processes are selected to recycle PGM, the first important operation is the leaching step. The platinum-group elements are generally leached with hydrochloric acid solutions containing chlorine or with aqua regia, in which the metals are dissolved as anionic chlorocomplexes such as $[PtCl6]^{2-}$. Therefore, extensive efforts are being made to find efficient, cost-effective, and environmentally friendly procedures aiming to the separation of these metals from acidic chloride media. Liquid–liquid extraction (LLE) is currently the most popular method used for this purpose (Bernardis *et al.*, 2005), since it offers a number of advantages over the classical precipitation methods. In fact, it exhibits a higher selectivity (Lee *et al.*, 2009a), as it involves scrubbing or stripping steps to achieve high metal purity, and allows the complete removal of metals by multi-stage extraction steps.

Extraction of metals by organic compounds dissolved in organic solvents has been gaining importance since the 1990s. Nevertheless, it has been reported that extraction (and separation) of PGM by LLE is a very difficult task because PGM chemistry is very similar (Sanuki *et al.*, 1999); the high price of PGM, the increasing demand for those metals in commercial and technological applications, their low amount in natural ores, and the deficit in PGM supplies have stimulated research on the use of LLE techniques to recover PGM from primary and secondary sources.

Some organic compounds for platinum extraction from spent samples or standard chloride media have been reported in the last years, and two main groups are generally found:

(i) organophosphorus compounds, such as tributylphosphate (TBP) (Sanuki *et al.*, 1999), 2-ethylhexylphosphonic acid mono-2-ethylhexyl ester (PC-88A) (Bandekar and Dhadke, 1998), tri-octylphosphine oxide (Cyanex 921) (Mhaske and Dhadke, 2001) and bis(2,2,4-trimethylpentyl)monothiophosphinic acid (Cyanex 302) (Kumar *et al.*, 2008);

(ii) amines, such as triisooctylamine (Alamine 308) (Lee *et al.*, 2009a), tri-octylamine (Alamine 336) (Sanuki *et al.*, 1999), other mixtures of tri-octylamine and tri-decylamine (Nowottny *et al.*, 1997) and ammonium quaternary salts such as Aliquat 336 (Lee *et al.*, 2009b).

Within these two groups, amines are more adequate, since platinum can be extracted without the interference of metals such as Fe and Al (Sanuki *et al.*, 1999) and, in addition, the organophosphorus compounds are not so environmentally friendly due to their incomplete incinerability (Thiollet and Musikas, 1989; Malik and Paiva, 2010).

In the last decade the interest for amide derivatives as extractant agents to recover platinum from concentrated chloride media has been growing, and compounds such as the following N,N′-tetrasubstituted malonamides have been extensively investigated for that purpose: N,N′-dimethyl-N,N′-dicyclohexylmalonamide (DMDCHMA), N,N′-dimethyl-N,N′-diphenylmalonamide (DMDPHMA), N,N′-dimethyl-N,N′-dibutylmalonamide, (DMDBMA), N,N′-dimethyl-N,N′-dihexylmalonamide (DMDHMA), N,N′-dimethyl-

N,N′-diphenyltetradecylmalonamide (DMDPHTDMA) and N,N′-dimethyl-N,N′-dicyclohexyltetradecylmalonamide (DMDCHTDMA). Although the extraction efficiency is highly dependent on the N,N′-substituent groups, those malonamide derivatives generally proved to be effective for platinum (IV) extraction from hydrochloric acid media, showing fast extraction kinetics and being easily usable. For DMDBMA and DMDHMA, Pt(IV) extraction increases with the HCl concentration in the aqueous phase, reaching values higher than 65% (Paiva *et al.*, 2014a, 2012). The best performance shown by DMDCHMA toward Pt(IV) extraction is less than 30% from 8 M HCl (Malik and Paiva, 2009), while DMDPHMA does not extract Pt(IV) at all. Pt(IV) extraction by DMDPHTDMA is very similar to the one presented by DMDCHMA (close to 30%) (Malik and Paiva, 2009). However, if tin (II) chloride is added to the aqueous phase as a labilizing agent, Pt(IV) extraction improves up to 90% (Malik and Paiva, 2009). Thus, by changing the structure of the malonamide derivatives it was possible to enhance the recovery of Pt(IV), as was the case of DMDCHTDMA; accordingly, 88% and 99% of Pt(IV) extraction from 6 M HCl and 8 M HCl, respectively, were achieved, without the addition of a labilizing agent (Costa *et al.*, 2013).

Functionalized amides containing pyridine, thioether, and secondary or tertiary amine groups in the structure have also been reported as highly efficient and selective extractants of PGM (Narita *et al.*, 2006). Thiodiglycolamide derivatives such as N,N′-dimethyl-N,N′-dicyclohexylthiodiglycolamide (DMDCHTDGA) exhibit a very good extraction ability for Pt(IV) from hydrochloric acid solutions (about 97% of Pt(IV) are extracted from 6 M HCl) (Paiva *et al.*, 2014b).

Another example of diamide extractants now considered for Pt(IV) extraction are N,N′-tetrasubstituted succinamides. Derivatives of this family have very recently been tested for the extraction of iron(III) (Cui *et al.*, 2015), as well as for actinides and lanthanides (Rapko *et al.*, 1999). The idea of using succinamides instead of malonamides for Pt(IV) extraction is based on the longer distance between the carbonyl groups of these molecules, due to the existence of an additional carbon atom placed between them, and on the hypothesis that such a structure could theoretically be more suitable to “accommodate” the high sized platinum extracted species. This hypothesis is also supported by evidence that an ion pair mechanism, possibly involving the oxygen protonation of the carbonyl group, is likely to be responsible for the extraction of Pt(IV) by N,N′-tetrasubstituted malonamides (Paiva *et al.*, 2014a). The work described in the present paper is part of a wider research project aiming to find out and select the most adequate extractants to recover PGM from solutions resulting from the leaching of secondary sources. Moreover, it reports for the first time investigation aiming to test the capabilities of succinamides for Pt(IV) extraction.

2. EXPERIMENTAL

2.1 Synthesis

FTIR spectra were recorded in a Bruker (Tensor 27) spectrophotometer, in KBr pellets. Melting points were measured on a SRS EZ-Melt Automated melting point apparatus. ^{1}H NMR spectra were acquired on a Bruker Avance 400, 400 MHz spectrometer, using $CDCl_3$ as solvent. The LC–MS equipment was composed by an Agilent Technologies 1200 Series LC coupled to a Bruker Daltonics HCT ultra (ion trap detector). The ionization was made by electro-spray (ESI) in the positive polarity. Typical ESI conditions were: nebulizer pressure 50 psi, capillary voltage 4.0 kV, and drying gas temperature 300°C. A GraceSmart column was used (100 mm length, 2.1 mm internal diameter, C18, 3 µm), stabilized at 25°C. The composition of the mobile phase was 25% water/75% MeOH, both with 0.1% formic acid, and the flow was 0.4 mL/min. The fragmentation spectra (MS^2) were obtained using Auto MS(n). High resolution mass spectra were obtained by electrospray ionization (ESI) in positive mode on a Bruker FTMS APEXIII spectrometer at Centro de Apoyo Científico-Tecnológico a la Investigación (C.A.C.T.I.), University of Vigo, Spain. The reagents and solvent for the synthesis, succinyl chloride (Sigma-Aldrich, 95%), N-methylcyclohexylamine (Sigma-Aldrich, 99%), N-methylaniline (Merck, ⩾98%) and dichloromethane (DCM, Absolve ⩾99%) were used without further purification.

The syntheses of N,N′-dicyclohexyl-N,N′-dimethylsuccinamide (DMDCHSA) and N,N′-dimethyl-N,N′-diphenylsuccinamide (DMDPHSA) were carried out by an adapted procedure (Walker, 1980) as follows: succinyl chloride (7 g, 5 mL, 0.044 mol) was dissolved in 75 mL of DCM, in a two-neck round-bottomed flask placed in a cold bath (ice/$CaCl_2$, −15°C). For the synthesis of DMDCHSA, N-methylcyclohexylamine (20.83 g, 24 mL, 0.184 mol) was dissolved in 25 mL of DCM, and added dropwise to the flask by a dropping funnel, under stirring, while for the synthesis of DMDPHSA, N-methylaniline (19.8 g, 20 mL, 0.185 mol) was added in the same manner. The reaction course was monitored by

FTIR, following the disappearance of the band at 1783 cm^{-1} (acyl chloride carbonyl group) and the appearance of the amide carbonyl band at 1639 cm^{-1} in the former case, or 1650 cm^{-1} for the latter. Both reactions attained completion after the end of the addition. The reaction mixtures were allowed to stir overnight and then washed three times with a 4% (v/v) HCl solution and one time with distilled water. The organic phase was dried over anhydrous magnesium sulfate, filtered to a round bottomed flask and evaporated in a rotary evaporator.

DMDCHSA and DMDPHSA were recrystallized from n-hexane and ethyl acetate, respectively, and dried under vacuum at 40°C overnight. 12.1 g of DMDCHSA and 10.4 g of DMDPHSA were obtained as white crystals. The overall data achieved for the characterization of both compounds is depicted in Table 3-1.

Table 3-1 - Characterization of *N,N'*-dicyclohexyl-*N,N'*-dimethylsuccinamide (DMDCHSA) and *N,N'*-dimethyl-*N,N'*-diphenylsuccinamide (DMDPHSA)

	DMDCHSA	**DMDPHSA**
Yield (%)	89	80
m.p. (ºC)	86-88	156-157
FTIR ($\bar{\nu}_{max}$/cm^{-1})	1639 (ν C=O), 1591 (Amide II)	1650 (ν C=O), 1594 (Amide II)
^{1}H NMR (δ/ppm)	4.44 (1 H, bs, CH cyclohexyl), 3.69 (1 H, bs, CH cyclohexyl), 2.88 (3 H, s, N-CH_3), 2.81 (3 H, s, N-CH_3), 2.73-2.65 (4 H, m, O=C-CH_2-CH_2-C=O), 1.85-1.05 (20 H, m, CH_2 cyclohexyl)	7.36-7.17 (10 H, m, phenyl), 3.18 (6 H, s, N-CH_3), 2.27 (4 H, s, O=C-CH_2-CH_2-C=O)
MS (*m/z*)	309 ($[M+H]^+$), 331 ($[M+Na]^+$), 639 ($[2.M+Na]^+$)	297 ($[M+H]^+$), 319 ($[M+Na]^+$), 615 ($[2.M+Na]^+$)
MS/MS (*m/z*)	196 ($[M-C_6H_{11}NHCH_3]^+$, 100%), 114 ($[C_6H_{11}NH_2CH_3]^+$, 10%)	190 ($[M-C_6H_5NHCH_3]^+$, 100%)
HRMS (*m/z*)	Calculated for $C_{18}H_{33}N_2O_2^+$ $[M + H]^+$ 309.25365; found 309.25322	Calculated for $C_{18}H_{21}N_2O_2^+$ $[M + H]^+$ 297.15975; found 297.15940

bs - broad singlet; s - singlet; m – multiplet.

2.2 Solvent Extraction Experiments

All reagents and solvents were used without further purification. 5 × 10^{-4} M Pt(IV) solutions were prepared from the $PtCl_4$ salt (Aldrich, 98%), which was previously dissolved in hydrochloric acid (37%, AnalaR NORMAPUR) solutions ranging from 1 to 8 M. Solutions with 1 × 10^{-3} M and 2.6 × 10^{-3} M of Pt(IV) in 6 M HCl, respectively, were also prepared, to be used for the extraction isotherm experiments.

The organic phases containing 0.05 M DMDCHSA or 0.05 M DMDPHSA, or other desired concentrations, were generally prepared in 1,2-dichloroethane (1,2-DCE, Fluka, minimum 99.5%), although other diluents were also used when the diluent effect was investigated: toluene (Panreac, minimum 99.5%), xylene (analytical reagent, Lab-Scan, minimum 99%, mixture of isomers), kerosene (Sigma–Aldrich, boiling point 175–325°C), dichloromethane (Riedel-de Haën, analytical reagent, minimum 99.8%), Escaid 100® (Exxon Mobil, boiling point 195–245°C, 18–24% aromatic content), Varsol 80® (Exxon Mobil, boiling point 206–238°C, 24% aromatic content), Exxsol D80® (Exxon Mobil, boiling point 208–236°C (0.2 wt% aromatic hydrocarbon content), and Shellsol D70® (Shell Chemicals, boiling point 198–242°C, low aromatic content).

Distilled water, 1 M HCl, seawater, 20 g L-1 chloride solution (as NaCl) and a solution composed by sodium lactate (6 g L-1), yeast extract (1 g L-1), trisodium citrate dihydrate (0.3 g L-1), ascorbic acid (0.1 g L-1) and thioglycolic acid (85.3 mg L-1) (designated "nutrient medium") were used as agents to strip platinum from the loaded organic phases.

General extraction and stripping experiments were carried out in duplicate at room temperature by contacting equal volumes of the organic and aqueous phases (A/O = 1) for 30 min, by mechanical stirring, adopting a rotation speed between 900 and 1000 rpm. After separation of the two phases, the aqueous phases were filtrated and their platinum content determined by flame-Atomic Absorption Spectrometry (AAS, Analytikjena NovAA 350 model spectrometer). The analysis of the solutions before and after extraction was performed in triplicate. The coefficient of variance determined for the results achieved from the different extraction and stripping experiments is approximately ± 5%.

3. RESULTS AND DISCUSSION

3.1 Synthesis of DMDCHSA and DMDPHSA

The structures of the two succinamide derivatives synthesized and investigated for.Pt(IV) extraction - *N,N'*-dicyclohexyl-*N,N'*-dimethylsuccinamide (DMDCHSA) and *N,N'*-dimethyl-*N,N'*-diphenylsuccinamide (DMDPHSA) - are presented in Figure 3-1.

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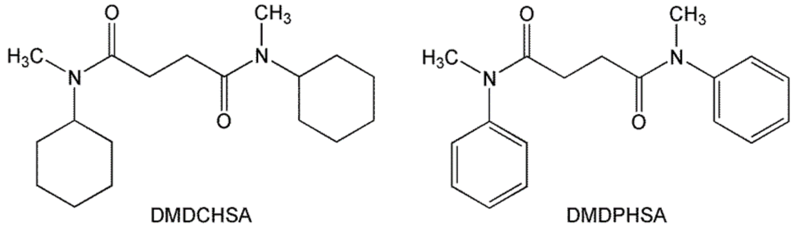

Figure 3-1- Chemical structures of DMDCHSA and DMDPHSA.

Both products were characterized by melting point, FTIR, ^1H NMR, and LC-MS (data summarized on Table 3-1), the spectroscopic data being consistent with the expected structures.

3.2. Solvent extraction experiments

All the extraction and stripping results presented were obtained in a single contact between the aqueous and the organic phase.

3.2.1. Effect of Hydrochloric Acid Concentration on the Extraction of Platinum (IV)

These experiments were performed using 0.05 M DMDCHSA and 0.05 M DMDPHSA, both in 1,2-DCE as organic phase, and varying the hydrochloric acid concentration in the aqueous media between 1 and 8 M. The extraction results obtained are displayed in Figure 3-2.

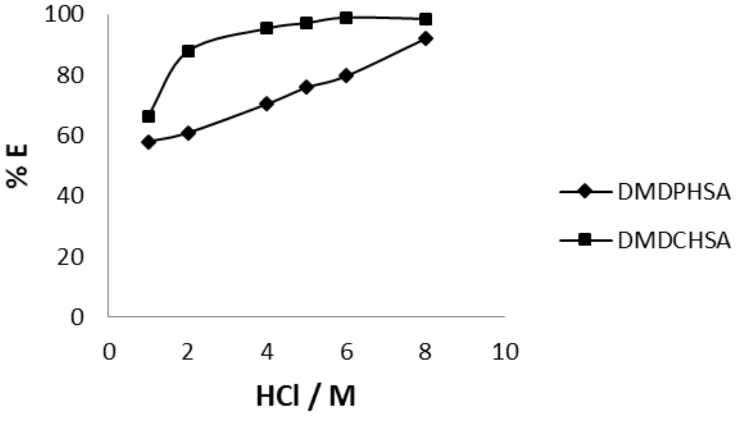

Figure 3-2 - Effect of HCl concentration on Pt(IV) extraction by 0.05 M DMDCHSA and 0.05 M DMDPHSA solutions in 1,2-DCE. [Pt(IV)] = 5 x 10^{-4} M. Standard deviations: ± 5%.

Pt(IV) extraction increases with increasing hydrochloric acid concentration in the aqueous phases for both compounds. However, DMDCHSA shows a more efficient Pt(IV) extraction behaviour for all HCl concentrations. Extraction percentages (%E) higher than 95% were obtained with DMDCHSA from 4 M HCl and more concentrated solutions. Pt(IV) extraction was almost complete from 6 M and 8 M HCl (%E values around 99%). With DMDPHSA, Pt(IV) extraction increases almost linearly but the %E achieved were generally lower than 90% (values of 58%, 70% and 80% were obtained from 1 M, 4 M and 6 M HCl solutions, respectively); nevertheless, the %E of Pt(IV) was 92% from 8 M HCl.

The increase of Pt(IV) extraction verified for the more concentrated HCl aqueous phases is a typical extraction behavior profile for similar diamide compounds, namely malonamides, such as DMDBMA (Paiva *et al.*, 2014a), DMDHMA (Paiva *et al.*, 2012), DMDCHTDMA (Costa *et al.*, 2013) and thiodiglycolamides (DMDCHTDGA) (Paiva *et al.*, 2014b). Pt(IV) extraction increases with the HCl concentration in the aqueous phase for DMDBMA, reaching %E of 91% at 6 M and 7 M HCl, while a similar but better profile was obtained for DMDHMA, which extracts Pt(IV) almost quantitatively above 5 M HCl (Paiva *et al.*, 2012). For DMDCHTDMA a similar profile was obtained: the %E Pt(IV) sharply boosts from 40% to 70% between 4 and 5 M HCl, reaching %E values of nearly 90% and 99% for 6 M and 8 M HCl, respectively (Costa *et al.*, 2013).

Malik and Paiva (2009) concluded that DMDPHTDMA shows a distinct extraction profile when compared with the ones previously mentioned, since it is only able to efficiently extract Pt in a single equilibration (%E > 90% for 6 M ≤ HCl ≤10 M) if tin(II) chloride is added to the aqueous phases.

The malonamides with structures similar to the succinamides described in this paper - DMDCHMA (*N,N'*-dimethyl-*N,N'*-dicyclohexylmalonamide) and DMDPHMA (*N,N'*-dimethyl-*N,N'*-diphenylmalonamide) were previously tested for the extraction of Pt(IV) under a similar set of experimental conditions (Costa *et al.*, 2013). Those malonamide derivatives do not efficiently extract Pt(IV): the maximum %E obtained for DMDCHMA at 8 M HCl was below 30%, while for DMDPHMA no LLE of Pt(IV) occurred. Therefore, it can be concluded that the presence of an additional carbon atom on the molecule structure is, in fact, determinant for the effectiveness of Pt(IV) extraction.

3.2.2. Effect of Equilibration Time on the Extraction of Platinum (IV)

Equilibration time is an important parameter that can affect metals extraction. Thus, the time necessary for the system to reach equilibrium was evaluated. For that purpose, a set of experiments was performed using 6 M HCl solutions containing 100 mg L-1 of Pt(IV) as aqueous phase, and 0.05 M DMDCHSA or

DMDPHSA in 1,2-DCE as organic phase, and different contact times ranging from 0.5 to 60 min were tested. The results presented in Figure 3-3 show a very fast rate of extraction, with 100% Pt(IV) extracted after 30 seconds for DMDCHSA and 81% for DMDPHSA. These very favorable extraction kinetics have also been observed for Pt(IV) extraction by DMDCHTDMA and DMDCHTDGA under the same conditions, where a contact time of 1 and 2 min resulted in 93% and 95% extraction, respectively, values equivalent to the ones achieved for longer periods of time (Costa *et al.*, 2013; Paiva *et al.*, 2014b).

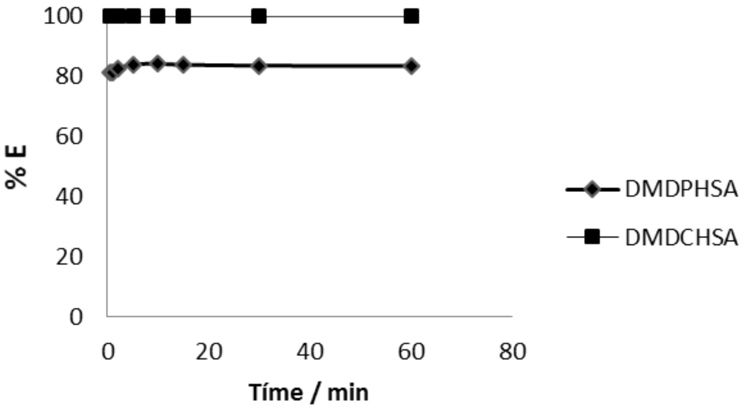

Figure 3-3 - Effect of contact time on Pt(IV) extraction from 6 M HCl by 0.05M DMDCHSA or DMDPHSA in 1,2-DCE. [Pt(IV)] = 5 x 10^{-4} M.

According to the data obtained, very short contact times, of a few minutes or even seconds, could be used for industrial purposes. This is an important result, as the need of long contact times to reach equilibrium can be a limiting factor for the viability of the metal recovery processes. Although much smaller contact times could be used, a contact period of 30 min was adopted for all the subsequent experiments, in order to assure enough time to reach the thermodynamic equilibrium of all the extraction systems tested.

3.2.3. Stripping of Platinum from the Loaded Organic Phases and Extractant Reutilization

Due to the good results attained for the extraction of Pt(IV) by the two succinamides tested, the investigation on the recovery of the metal from the loaded organic phases to new and purified aqueous media is a relevant issue for the success of the whole process.

The reasons behind the selection of the stripping agents were based on previous information concerning the stripping of Pt(IV) from organic phases in which similar structures, such as N,N´-tetrasubstituted malonamides, were used, and on the economy and availability of the stripping agents. Therefore, taking into account effectiveness considerations, HCl solutions were tested (Costa *et al.*, 2013). Both economy and efficiency determined that distilled water was also to be tested (iron(III) was quantitatively stripped from malonamide derivatives (Costa *et al.*, 2007, 2003)). Taking into account that chloride ions are most probably determinant for the efficiency of the stripping processes, and knowing that seawater is the most important and economic source of that anion, seawater was also tested. The great availability of seawater, particularly in coastal areas, was also taken into consideration. Pure NaCl solutions containing 20 g L-1, the typical chloride concentration in seawater, were tested for comparison. Finally, a more complex solution, designated as “nutrient medium”, already described in the experimental section (containing lactate and a number of other salts dissolved in seawater), was also evaluated. The use of such a solution, with typical bacterial nutrient compounds, aimed to the possibility of its subsequent inoculation with bacteria that allow the bio-reduction of platinum (Rashamuse and Whiteley, 2007; Konishi *et al.*, 2007), thus combining different promising technologies to achieve the final metallic platinum recovery.

In the stripping experiments, the organic phases (0.05 M DMDCHSA or DMDPHSA in 1,2-DCE) contacted previously with a 6 M HCl aqueous solution containing $5x10^{-4}$ M Pt(IV) at A/O= 1, for 30 min. Thereafter, the Pt loaded organic phase contacted with the solution of the stripping agent, again for 30 min and A/O= 1. The results obtained are summarized in Table 3-2.

Table 3-2 - Percentages of Pt stripping from loaded organic phases of DMDCHSA and DMDPHSA in 1,2-DCE (obtained from the extraction of $5x10^{-4}$ M Pt(IV) from 6 M HCl solutions) through the use of different stripping agents. (Standard deviation: ± 5%)

Stripping solution	**%S Pt**	
	DMDCHSA	DMDPHSA
1 M HCl	26	7
Distilled water	38	6
Seawater	99	58
“Nutrient medium”	40	18
20 g L^{-1} [Cl^-] solution (as NaCl)	35	19

Using distilled water, 1 M HCl, 20 g L-1 of chloride ion as NaCl, and the “nutrient medium”, Pt was only partially stripped from DMDCHSA, since the results varied from 26% for 1 M HCl to 40% for the "nutrient medium". Stripping of Pt from DMDPHSA is much less efficient: values between 6% and 19% were obtained with distilled water and with the 20 g L-1 NaCl solution, respectively. The best results were achieved with seawater for both extractants, 99% for DMDCHSA and 58% for DMDPHSA. According to the results, chloride ions do not seem to be the only key to obtain an efficient stripping. Other substances present in seawater (salts such as sulfates and organic matter) appear to make the difference to achieve good Pt(IV) stripping results.

Summarizing, it can be concluded that Pt can be almost totally stripped from the loaded DMDCHSA organic phase using just seawater. This result is promising considering that seawater is a largely available and economic resource, although, according to our knowledge, no data were previously reported about its use for this purpose. In the case of DMDPHSA, several contacts with seawater can eventually be performed to achieve a higher Pt stripping percentage than that obtained in a single contact.

Although the use of seawater was successful for Pt stripping, it should be mentioned that the presence of high concentrations of salts can be an inconvenient during the subsequent solution treatment to obtain the pure metal or salt. However, the research on new materials less affected by seawater corrosion (Hassan and Malik, 1989; Al-Malahy and Hodgkiess, 2003), as well as novel alternatives aiming to the subsequent recovery of the metal from the metal loaded stripping phases, such as biologic methods, can contribute to overcome possible problems. For instance, the literature mentions the use of several bacterial strains able to recover Pt from aqueous media (Konishi, *et al.*, 2007; Narayanan and Sakthivel, 2010; Riddin *et al.*, 2010). Our group also investigates the recovery of PGM by bacterial communities and some data, not yet published, showed that PtS_2 nanoparticles were obtained from a solution of seawater resulting from Pt stripping.

In order to collect information on the stability of DMDCHSA or DMDPHSA when reused, five successive extraction-stripping stages were carried out, using feed aqueous phases containing $5x10^{-4}$ Pt(IV) in 6 M HCl and seawater as stripping solution, always involving the same portion of 0.05 M DMDCHSA (or DMDPHSA) in 1,2-DCE. The results obtained in each extraction and stripping stage are represented in Figure 3-4.

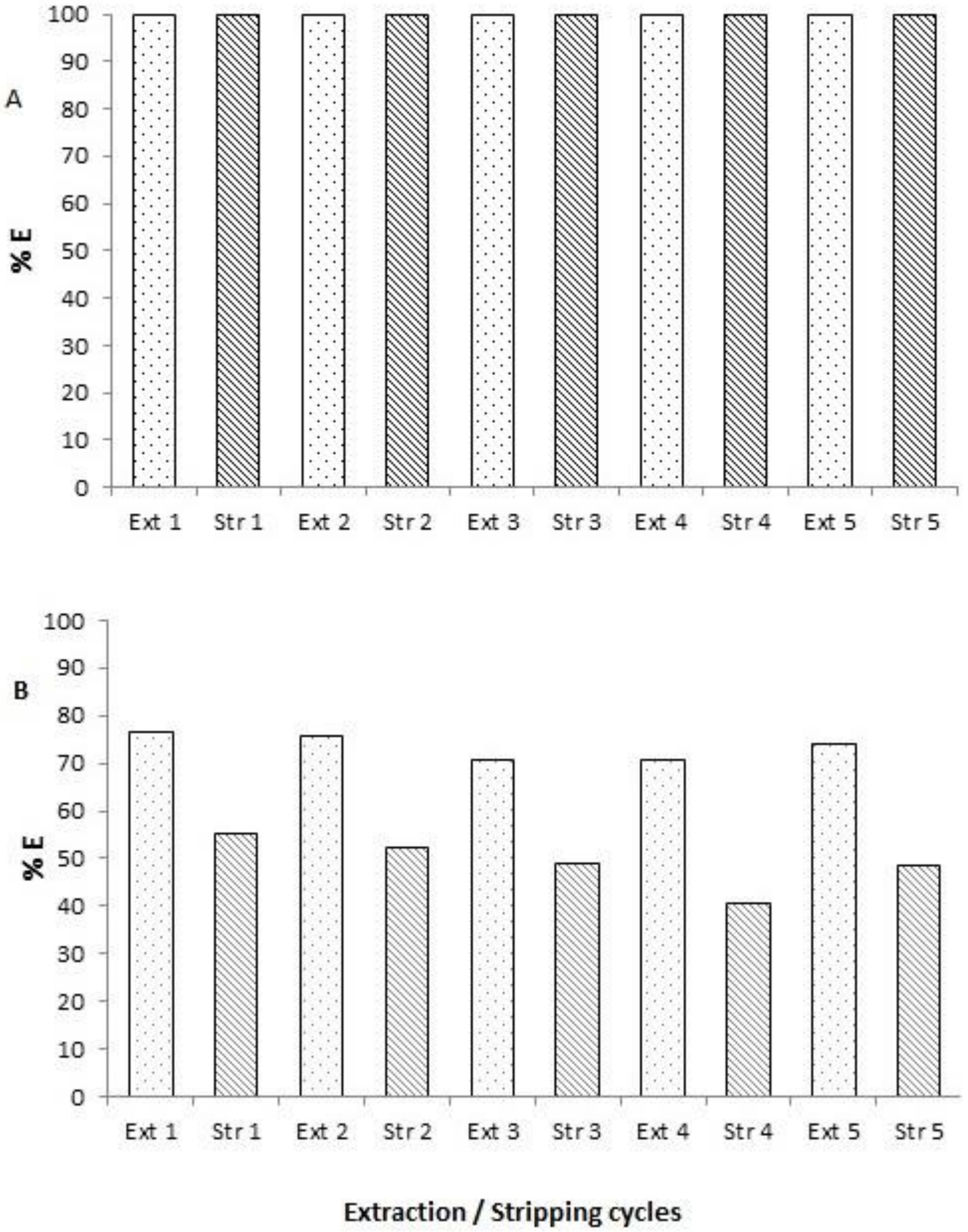

Figure 3-4 -Evaluation of 0.05 M DMDCHSA (A) and 0.05 M DMDPHSA (B) performance in successive extraction-stripping cycles. [Pt(IV)] = 5 x 10^{-4} M in 6 M HCl, extractants in 1,2-DCE; stripping with seawater. Standard deviations: ± 5%.

It can be concluded that the two extractants can be reused, though it should be stated that DMDCHSA has better outcomes than DMDPHSA in the five successive extraction-stripping stages. DMDCHSA always reached values near 100% in both extraction and stripping stages, while for DMDPHSA Pt(IV) extraction varied between 70% and 80%, and stripping from about 40% to 55%.

The results achieved with DMDCHSA in terms of reutilization are again very encouraging, bearing in mind an eventual industrial application.

3.2.4. Extraction Isotherms of Platinum (IV)

The loading capacity of DMDCHSA and DMDPHSA in 1,2-DCE was investigated by shaking an aqueous solution of 6 M HCl containing 2.6x10^{-3} M Pt(IV) with 0.05 M DMDCHSA or DMDPHSA and varying the ratio between the two phases, A/O, from 1 to 15. The results are displayed in Figure 3-5.

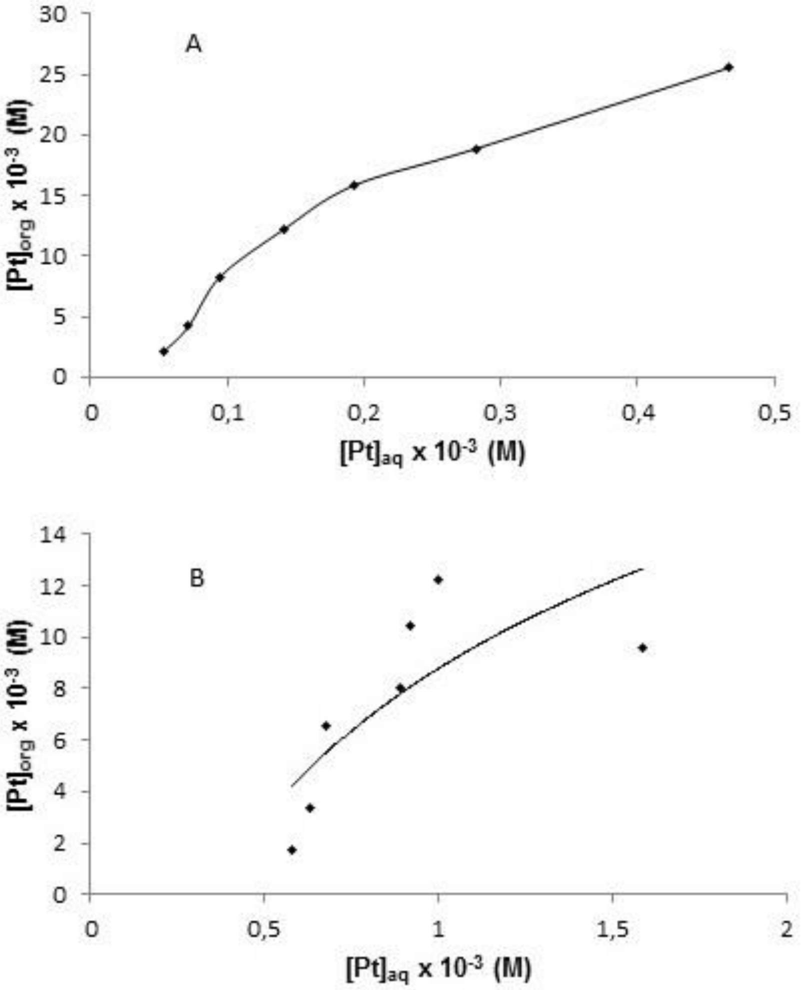

Figure 3-5 - Equilibrium extraction isotherms for Pt(IV) by 0.05 M DMDCHSA (A) and DMDPHSA (B) both in 1,2-DCE. [Pt(IV)] = 2.6 x 10^{-3} M in 6 M HCl. Standard deviations: ± 5%.

The information collected points out to loading capacities higher than 2.5 x 10^{-2} M Pt(IV) for DMDCHSA, and higher than 1.2 x 10^{-2} M Pt(IV) for DMDPHSA, since the Pt(IV) saturation plateaus for both extractants were not attained. The accumulated Pt(IV) concentration by DMDCHSA is already very high, corresponding to a DMDCHSA:Pt(IV) molar ratio of 2.0. The Pt(IV) concentration values achieved for DMDPHSA are lower than for DMDCHSA, but they also reveal a good loading capacity.

Both extractants present higher Pt(IV) loading capacities than DMDCHTDMA and DMDPHTDMA under similar experimental conditions: about 1.1 x 10^{-2} M for DMDCHTDMA, the extractant:Pt(IV) molar ratio being 4.5 (Costa *et al.*, 2013), and 7.19 x 10^{-4} M Pt(IV) for DMDPHTDMA, but in the presence of $SnCl_2$ (Malik and Paiva, 2009).

3.2.5. Effect of Different Diluents on the Extraction of Platinum (IV)

It is well known that chlorinated diluents are not suited for industrial application, due to their toxicity. Taking that into account, a variety of diluents was tested in order to assess the possibility to replace 1,2-DCE by a more environmentally friendly diluent. Aromatic diluents such as toluene and xylene (mixture of isomers), and commercial diluents such as kerosene, Escaid 100®, Varsol 80®, Exxsol D80® and Shellsol D70® were used for that purpose. The extraction experiments were carried out using a solution of 5x10^{-4} M of Pt(IV) in 6 M HCl and the extractant concentration (DMDPHSA and DMDCHSA) was kept constant at 0.05 M. Both extractants were in general more difficult to dissolve in those diluents than in 1,2-DCE or other chlorinated diluents, but complete dissolution was attained after sonication. The results obtained are displayed in Table 3-3.

Table 3-3 - Effect of different diluents on the extraction percentage of 5 x 10^{-4} M Pt(IV) in 6 M HCl by 0.05 M DMDCHSA and DMDPHSA (standard deviation: ± 5%)

Diluent	**% E Pt(IV)**	
	DMDCHSA	DMDPHSA
1,2-DCE	98	80
Dichloromethane	69	40
Toluene	16	33
Kerosene	16	30
Xylene (mixture of isomers)	14	34
Escaid 100®	4	29
Varsol 80®	6	36
Exxsol D80®	8	34
Shellsol D70®	13	41
1-Octanol	74	72

Unfortunately none of the diluents tested was better than 1,2-DCE. Dichloromethane was the best after 1,2-DCE, showing that probably the use of polar diluents is decisive to achieve efficient Pt(IV) extraction. The non-chlorinated diluents are not efficient for Pt(IV) extraction with both DMDPHSA and DMDCHSA. DMDPHSA presented in general better extraction results than DMDCHSA in almost all diluents tested, with exception for the chlorinated ones. Similar results were previously obtained for Pt(IV) extraction by DMDBMA and DMDCHTDMA, since Escaid 100®, Escaid 110®, and Shellsol D70® were also not efficient in those cases (Paiva *et al.*, 2014a; Costa *et al.*, 2013). According to the literature, the usual hydrocarbon diluents promote aggregation of the malonamide extractants (Berthon *et al.*, 2010), whereas polar diluents do not seem to favor this phenomenon. This justification can probably be also applied to succinamide extractants, as aggregation may likely inhibit Pt(IV) extraction by DMDCHSA and DMDPHSA when commercial diluents are used. Taking into account the results obtained, the use of a polar diluent, 1-octanol, more environmentally friendly than chlorinated diluents, was then tested. The percentage of Pt(IV) extraction from a 6 M HCl solution using 0.05 M DMDCHSA and 0.05 M DMPHSA, both dissolved in 1-octanol, was 74% and 72%, respectively, showing the importance of polar diluents to guarantee an efficient metal extraction by the succinamide derivatives. Subsequent experiments showed that 1-octanol alone can be used for Pt(IV) extraction from HCl media, since about 80% of %E were obtained using 1-octanol in the absence of the succinamides, whereas none of the other diluents showed that capacity. Further research is currently in progress to evaluate the Pt(IV) extraction ability shown by this alcohol derivative.

3.2.6. Effect of DMDCHSA and DMDPHSA Concentration on Platinum (IV) Extraction

This set of experiments was carried out with 5 x 10^{-4} M Pt(IV) in 6 M HCl aqueous solutions. DMDCHSA and DMDPHSA concentrations in 1,2-DCE were varied between 0.001 M and 0.1 M.

The %E Pt(IV) increases when higher extractant concentrations are used. For concentrations of DMDCHSA higher than 0.05 M the extraction was 100%. The log-log plot between Pt(IV) distribution ratios (*D*) and DMDCHSA concentrations indicates a slope value near to 1, as can be seen in Figure 3-6 A. Hence, this result could suggest that species bearing a 1:1 metal:extractant stoichiometry exist in the organic solutions. However, the slope of the three first values of the graph is 0.57, and a similar slope value of 0.64 was obtained for the last three values of the graph. According to this interpretation, a 2:1 metal:extractant ratio is more likely to occur, and thus a 2:1 metal:extractant species may exist in the organic phase. A similar behavior was observed for DMDPHSA, since a slope value of 0.43 was obtained (Figure 3-6 B). A metal:extractant ratio near 2 may be due to the existence of two possible metal binding sites in the succinamide molecules (the positively charged amide groups). The high Pt(IV) loading capacities presented by these two extractants can eventually be related to the ability of each succinamide molecule to be attached to two Pt(IV) ions. Variations of the extractant:Pt(IV) stoichiometries in the succinamides concentration range selected should also be taken into account, which can explain the slight different slope tendencies observed in the same graphs.

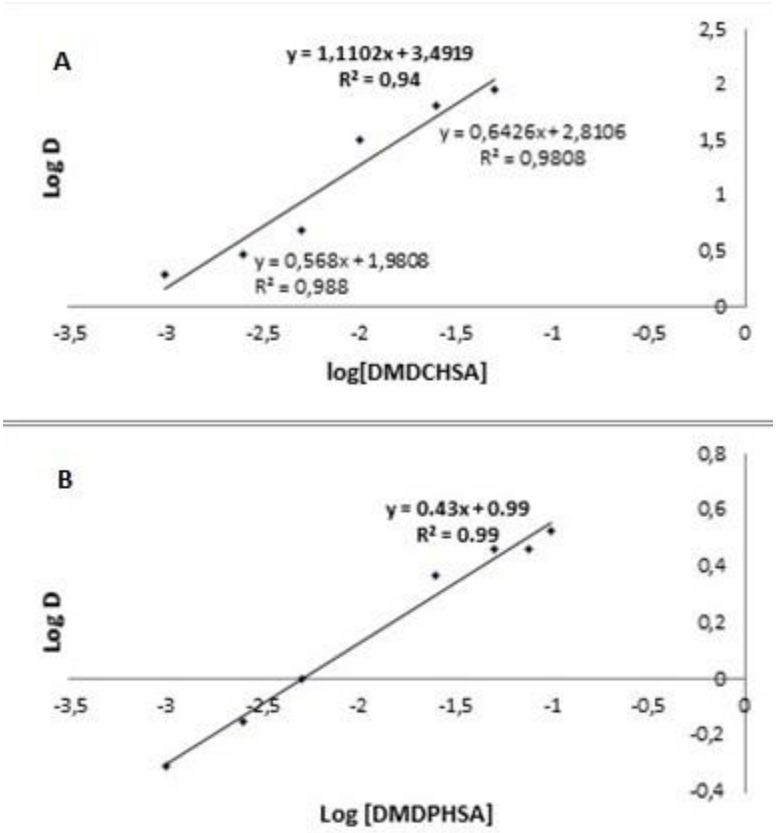

Figure 3-6 - Effect of DMDCHSA (A) and DMDPHSA (B) concentrations on Pt(IV) extraction from 6 M HCl. [Pt(IV)] = 5 x 10^{-4} M. Standard deviations: ± 5%.

The apparent contradiction between the loading results reported in section 3.2.4 and the data obtained herein may be firstly explained by the fact that the saturation plateaus were not effectively reached. Furthermore, although saturation plateaus provide clues about the extractant/metal ion proportions, diverse contributing phenomena can influence the saturation results, such as mass transfer, diffusion, aggregation of the extractant or the extracted species, and interfacial features. Hence, information from saturation plateaus can provide less, e.g. (Gupta and Singh, 2013), identical, or higher stoichiometries, e.g. (Costa *et al.*, 2013), than the slopes obtained from the log *D*-log [extractant] plots.

Different Pt(IV):extractant stoichiometries were previously obtained for malonamides and thiodiglycolamide derivatives in the same conditions (with 5 x 10^{-4} M Pt(IV) in 6 M HCl aqueous solutions and extractant concentrations in 1,2-DCE varying between 0.001 M and 0.1 M): for DMDCHTDMA the log-log plot indicates the existence of species bearing 1:1 and/or 1:2 metal:extractant stoichiometries in the organic solutions (Costa *et al.*, 2013) and for DMDCHTDGA the results suggest that species bearing a 1:1 Pt(IV):DMDCHTDGA stoichiometry should exist in the organic solutions (Paiva *et al.*, 2014b).

Taking into account the extremely favorable kinetics and the extraction profiles obtained when the HCl concentration was varied, it is likely that Pt(IV) may be extracted through ion-pairs (Preston and du Preez, 2002), at least for HCl concentrations above 4 and 5 M. However, in order to be sure about the composition of the Pt(IV) extracted species by the two succinamide derivatives, additional investigation would be necessary, namely the thorough study of the effects caused in the Pt(IV) distribution ratio when chloride and hydrogen ion concentrations are varied in the aqueous media, along with spectroscopic and X-ray crystallography data.

4. CONCLUSIONS

This article demonstrates the potentialities of N,N′-tetrasubstituted succinamides – N,N′-dicyclohexyl-N,N′-dimethylsuccinamide (DMDCHSA) and N,N′-dimethyl-N,N′-diphenylsuccinamide (DMDPHSA) – as Pt(IV) solvent extraction molecules for the first time. In fact, these succinamides are promising extractants for the recovery of platinum (IV) from hydrochloric acid media, since it was found that Pt(IV) extraction increases with HCl concentration: values higher than 95% were reached from 4 M or higher HCl concentrations with

DMDCHSA, while %E of 80% and 92% were achieved with DMDPHSA from 6 M and 8 M HCl, respectively.

Pt(IV) can be efficiently stripped to a new aqueous phase by a simple contact of the loaded organic phases with seawater. The Pt(IV) extraction processes are kinetically favorable, and both DMDCHSA and DMDPHSA show quite good loading capacities toward Pt(IV). In addition, these extractants can be reutilized several times without losing their extraction ability. However, when 1,2-DCE is replaced by more environmentally friendly diluents, Pt(IV) extraction is negatively affected.

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CHAPTER 4

Palladium recovery as nanoparticles by an anaerobic bacterial community

ABSTRACT

BACKGROUND: Biorecovery of metals from dilute industrial waste using bacteria is a promising alternative to primary raw material resources. Therefore, the search for and identification of palladium resistant bacteria with the ability to remove that metal from solutions is very important. The main goal of this study was to find a palladium (II) resistant bacterial community able to biorecover this metal from solution.

RESULTS: A palladium (II) resistant bacterial community able to precipitate that metal from the growth medium was obtained from a sludge sample from a municipal waste water treatment plant. This community was able to remove 60% of palladium (II) from an aqueous solution containing 18 mg L^{-1} of Pd(II) and 43% in the presence of 26 mg L^{-1} Pd(II) plus sulphate. TEM-EDS analysis showed the presence of nanosized palladium deposits in the surface of cells. Phylogenetic analysis of the 16S rRNA gene showed that this community was mainly composed of bacteria closely related to several *Clostridium* species. However, bacteria affiliated to genera *Bacteroides* and *Citrobacter* were also present in the community.

CONCLUSION: The current study for the first time reports the biorecovery of Pd(II) as nanoparticles by a bacterial consortium, and is a relevant demonstration of the biotechnological potentialities of this community.

Keywords: Biorecovery, Palladium, Nanoparticles; Bacterial community

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1. INTRODUCTION

As a result of their high value, the rising demand and the progressive depletion of primary raw material resources, the recovery and reutilization of platinum group metals – PGM: e.g. palladium (Pd), platinum (Pt), rhodium (Rh) and ruthenium (Ru) – from industrial wastes has been encouraged.

Several chemical methods have been explored to recover PGM, namely chemical reductive (Konishi *et al.*, 2007) and electrochemical methods (Dean, 1979). However, these techniques can be expensive, polluting and/or inefficient for PGM recovery from exhausted or dilute effluents from mines or industry. Biorecovery strategies using microorganisms have been considered a clean and economic alternative for metal recovery at low concentrations (Macaskie *et al.*, 2010). Microorganisms can be used to recover metal ions from aqueous solutions through several mechanisms. Biological reduction of Pd(II) to Pd(0) by pure cultures of *Desulfovibrio desulfuricans* (Yong *et al.*, 2002) and *Shewanella oneidensis* (De Windt *et al.*, 2005) has been demonstrated, while Vargas and colleagues (2004) revealed the bioabsorbance of Pd and Pt by *Desulfovibrio desulfuricans*. Bioaccumulation of palladium by a wild-type *Desulfovibrio fructosivorans* has also been observed by Mikheenko and colleagues (2008).

Although there are several studies concerning the recovery of Pd(II) using pure cultures, its applicability is limited. The main causes are related to the use of sterile conditions to prevent external microbial contamination which increase the process cost and to the difficulty maintaining the culture and its performance in the long term, due to the higher susceptibility to even small variations in experimental conditions. For these reasons, results obtained by mixed cultures are in general more relevant and realistic for the optimization, design and operation of these biological recovery systems. In that context, the reduction of Rh(III) and Pt(IV) by mixed consortia of sulphate-reducing bacteria (SRB) has already been demonstrated (Rashamuse and Whiteley, 2007). Besides the potential of microorganisms in terms of biorecovery of metals, their ability to produce metallic nanoparticles (NPs) is an extra-value considering their unique properties such as high corrosion resistance and stability to oxidation at high temperatures (Lloyd *et al.*, 2005). These properties are responsible for the vast application of NPs, which includes catalysis (Moreno-Manas and Pleixats, 2003), biosensing (Han *et al.*, 2001), biological labelling (Nicewarner-Pena *et al.*, 2001), electronics (Kamat, 2002), optical devices (Rao and Cheetham, 2001) and control drug delivery (De Windt *et al.*, 2006). Metal NPs have been produced by several pure bacteria cultures including *Desulfovibrio desulfuricans Desulfovibrio fructosovarans, Desulfovibrio vulgaris, Shewanella oneidensis, Bacillus sphaericus, Citrobacter braakii* and *Clostridium* pasteurianum (Yong *et al.*, 2002; Vargas *et al.*, 2004; Pollmann *et al.*, 2006; Baxter-Plant *et al.*, 2003; De Corte *et al.*, 2012).

As mentioned above, although the use of mixed bacterial cultures presents advantages over pure cultures (Mukred *et al.*, 2008), few studies have focused on PGM recovery using mixed cultures and to our knowledge their use was never reported for Pd recovery. Thus, in the present study a sulphate-reducing bacteria enriched consortium was used to evaluate its palladium (II) removal ability. In addition to its potential in terms of biorecovery of palladium, its ability to produce metallic NPs was also investigated. The phylogenetic characterization of the bacteria involved in palladium recovery was included as well. This study hopes to contribute to the development of low cost and environmentally acceptable biorecovery and bio-synthesis procedures of palladium nanoparticles.

2. MATERIAL AND METHODS

2.1. Microorganisms and growth conditions

The anaerobic bacterial community used in the present study was selected from previous works and contained the SRB species: *Desulfovibrio desulfuricans* and *Desulfobulbus rhabdoformis* (Martins *et al.*, 2009). This consortium was enriched from a sludge sample from a municipal waste water treatment plant located in Montenegro, Faro, in southern Portugal (Martins *et al.*, 2009; Martins *et al.*, 2012). Stock cultures were maintained in Postgate B medium (Postgate, 1984) supplemented with resazurin as redox indicator (0.03 g L^{-1}), at room temperature (21 ± 2°C) in anaerobic conditions. Subsequently, the bacterial mixed culture was grown in palladium test medium (PdTM) which contains, 1 g L^{-1} NH4Cl, 0.5 g L^{-1} KCl, 0.06 g L^{-1} $CaSO_4.6H_2O$, 1 g L^{-1} yeast extract, 0.06 g L^{-1} $MgCl_2{\cdot}6H_20$, 2 g L^{-1} Na_2SO_4, 6 g L^{-1} sodium lactate and 5 mg L^{-1} of Pd(II) as palladium (II) chloride. The culture was sub-cultured every 4 weeks using 10% (v/v) inoculum and the bacterial growth was monitored by weekly determination of pH, Eh and sulphate concentration.

2.2. Batch Experiments

The assays were performed in batch mode under anaerobic conditions, using the palladium test medium (PdTM) previously described (pH=7.5 ± 0.2). All experiments were performed in duplicate using glass bottles (120 or 35 mL) containing 100 or 30 mL of PdTM and 10% (v/v) of inoculum. The bacterial cells obtained previously were harvested by centrifugation at 4000 rpm for 10 min, washed with growth medium and transferred to the bottles containing the medium to be tested. The medium was purged with nitrogen gas to achieve an anaerobic environment prior to inoculation and about 10 mL of paraffin was then added. Finally, the bottles were sealed with butyl rubber stoppers and aluminium crimp seals and incubated at room temperature (21 ± 1◦C).

2.2.1. Palladium (II) removal by live cells

The studies of Pd(II) bioremoval were performed in 120 mL glass bottles containing PdTM, previously described, supplemented with palladium (II) at range concentrations from 6 to 27mg L^{-1}. For each experiment an abiotic control was carried out in parallel. The abiotic controls were prepared in the same way as the biotic tests, but without inoculum addition.

The ability of palladium (II) removal from the medium by the bacterial culture was also investigated in the absence of sulphate. In that case the calcium sulphate salt was replaced by calcium chloride ($CaCl_2.6H_2O$) and the sodium sulphate was removed from the medium.

2.2.2. Pd(II) removal by heat-killed cells

Palladium (II) bioremoval by heat-killed cells was also explored using 35 mL glass bottles. Bacterial cells (30 mL) were harvested by centrifugation at 4000 rpm for 10 min and washed with growth medium. The cells were killed by autoclaving (121◦C, 30 min) and added to bottles containing PdTM with 15.0 ± 0.5 mg L^{-1} of palladium (II).

2.3. TEM-EDS and XRD analyses

The precipitates generated during the bioremoval process were characterized by X-ray powder diffraction (XRD) using aPANalytical X'Pert Pro powder difractommeter operating at 45 kV and 40 mA, with CuKα radiation filtered by Ni. XRD patterns were recorded using a X'Celerator detector, with a step size of 0.016 and a time per step of 50 s. The HighScore Plus software, with the ICDD PDF-2 database, was used for peak analysis and crystalline phase identification.

Transmission electron microscopy coupled with an energy dispersive spectrometer (TEM-EDS, Hitachi H8100) (concept of the technique in Appendix 3) was used to establish the localization of the metal precipitates in the cells and the elemental characterization of the metal deposits. Samples of fresh bacterial cells exposed to 18 mg L^{-1} palladium (II) in the presence and absence of sulphate were prepared for TEM by fixation with glutaraldehyde 3% followed by dehydration and embedding in Epon-Araldite (Glauert, 1975). Thin sections (79–90 nm) without staining were used for detection of electron-dense precipitates. The precipitates were analysed at 200 kV using an EDAX EDS detector. For characterization of particle ultra-structure, particle suspensions in distilled water, were adsorbed onto carbon membrane coated copper grids and observed in JEOL 100SX and/or Hitachi H8100 transmission electron microscopes.

2.4. Analytical methods

Periodically, samples from cultures were collected using a syringe and optical density at 600 nm (OD_{600}) was measured in each sample. The samples were analyzed after centrifugation at 4000 rpm for 5 min. Redox potential and pH were determined using a pH/E Meter (GLP 21, Crison). Sulphate concentration was quantified by UV/visible spectrophotometry (Hach-Lange DR2800 spectrometer) using the method of SulfaVer®4. Palladium (II) removal was monitored by flame atomic absorption spectroscopy (AAS, Varian Spectrometer model AA-20). In palladium analysis, for each sample five readings were considered and the results were critically treated and only accepted with a reasonable standard deviation (<2%). Gaseous samples from head-space of the bottles were collected at the end of the experience and immediately analyzed by gas chromatography (GC). Subsequently, the total pressure was measured to calculate the total amount of H_2 in the gas phase.

2.5. Molecular characterization

Extraction of DNA, PCR amplification and cloning of 16S rRNA gene Total genomic DNA was extracted after harvesting cells by centrifugation at 4000 rpm for 10 min. DNA extraction was carried out as described by Martins and colleagues (Martins *et al.*, 2010b). Amplification of full-length 16S rRNA gene was performed using the primer pair 8F (5’- AGA GTT TGA TCC TGG CTC AG - 3’) / 1492R (5’- GGT TAC CTT GTT ACG ACT T -3’) (Suzuki *et al.*, 2003) according to conditions previously described (Martins *et al.*, 2010b). The PCR products were analyzed by electrophoresis, in 1% (w/v) agarose gel and TAE buffer. The band with the proper size range (approximately 1.4 kb) was excised and purified with E.Z.N.A.TM Gel Extraction Kit (Omega). The purified products were ligated into the cloning vector pGEM®-T Easy according to the manufacturer's instructions (Promega, Madison, USA), followed by transformation into *Escherichia coli DH5a* competent host cells. The white colonies were screened for inserts by amplification with a vector specific primer set (Sp6 and T7) according to conditions previously described (Martins *et al.*, 2010b). The PCR products were analyzed by electrophoresis, in 1% (w/v) agarose gel and TAE Buffer and the clones containing expected DNA insert were saved at −20◦C.

2.5.1. Restriction fragment length polymorphism analysis (RFLP) of 16S rRNA gene and phylogenetic analysis

RFLP analysis of the previously amplified 16S rRNA gene was performed using the restriction enzymes *HhaI* and *MspI* (Promega) to search for similar rRNA gene clones using the methodology previously described (Martins *et al.*, 2010b). Sequence identification was performed by use of the BLASTN facility of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences obtained in this study have the following accession numbers: JX273749 to JX273760. Phylogenetic trees were constructed using MEGA 4 and the Neighborhood-Joining algorithm was applied (Saitou and Neil, 1987; Studier and Keppler, 1988).

3. RESULTS AND DISCUSSION

Although scarce due to its low concentration in the Earth crust, palladium request increased exponentially over the last century mainly due to its high catalytic activity, which is exploited in many industrial processes. Therefore, its recovery from industrial wastes has been considered an attractive alternative, which requires the development of efficient and low cost processes.

Biotechnological approaches based on the bacterial activity have been considered an efficient recovery technique for palladium and other metals (De Corte *et al.*, 2012). Several microorganisms with ability to remove Pd(II) from solutions have been reported (De Corte *et al.*, 2012). However, despite the advantages of using mixed cultures (Martins *et al.*, 2010a), the recovery of palladium from aqueous solutions by bacterial communities was never reported. In this paper, the ability for palladium (II) removal by a SRB enriched community was tested. This community has shown an excellent metal resistance20 and consequently can be a potential candidate for palladium (II) removal from aqueous media.

3.1. Pd(II) bioremoval

Bacterial growth and palladium removal in the presence of sulphate and different concentrations of Pd(II): 6 mg L^{-1} (a), 10 mg L^{-1} (b), 18 mg L^{-1} (c) and 26 mg L^{-1} (d) are shown in Figure 4-1. The bacterial growth was similar in the presence of 6 and 10 mg L_{-1} of palladium (II) achieving an OD_{600} of 0.65 and 0.60, respectively, after 6 days of incubation. Although the lag phase was higher, bacterial growth achieved a similar OD_{600} value (0.67) in the presence of 18 mg L^{-1} of metal at the end of the experiment. For the highest palladium concentration (26 mg L^{-1}) bacterial growth was comparatively slower and thus a smaller OD_{600} value (0.49) was achieved at the end of the experiment. Besides the palladium (II) resistance, the bacterial community showed ability to remove Pd(II) from solution for all metal concentrations tested, although a longer adaptation phase was observed for the highest concentration (26 mg L^{-1}) (Figure 4-1(d)), which is in accordance with the bacterial growth profile. Approximately 60% of palladium (II) was removed from the solution after 13 days of incubation in the presence of 6 mg L^{-1} and after 6 days in the presence of 10 and 18 mg L^{-1} of palladium (II). In the presence of 26 mg L^{-1}, 43% of palladium (II) was removed after 26 days of incubation.

Although SRB mixed culture showed excellent sulphate reducing performance before palladium (II) exposure, sulphate reduction was affected when palladium (II) was added to the medium (Figure 4-1). Sulphate removal decreased from 60% to less than 40% when Pd(II) concentration was increased from 6 to 10 mg L^{-1} and for higher concentrations no sulphate removal was observed. This behaviour could be explained by the dynamics of the consortium, which probably was modified by selection driven by the presence of Pd(II). In fact, it has already been demonstrated that modifications to bacterial growth conditions can severely modify the bacterial population (Martins *et al.*, 2010b; Assunção *et al.*, 2011).

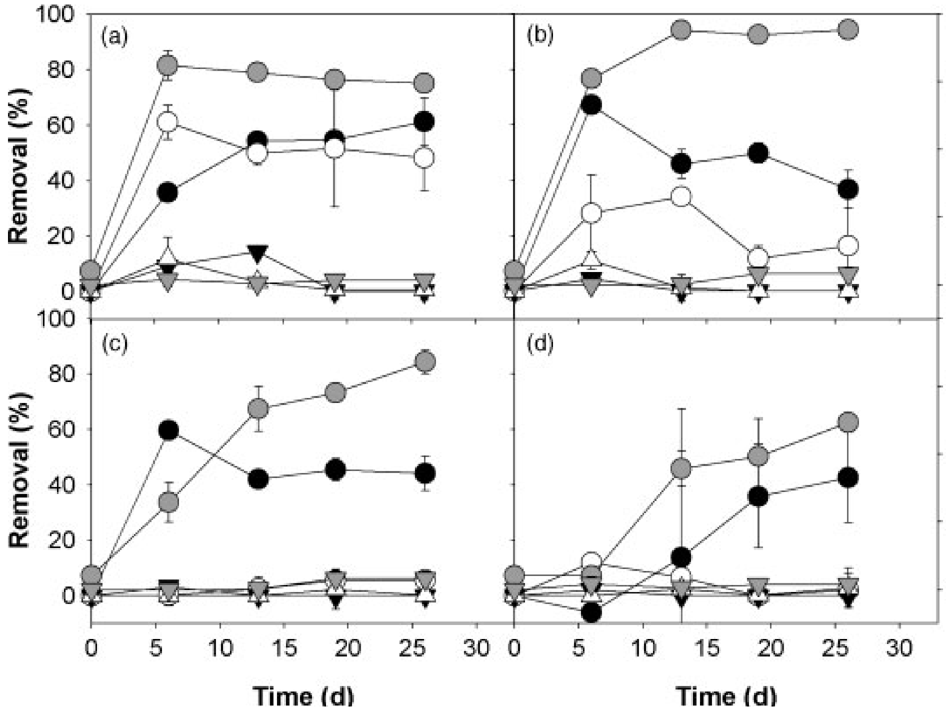

Figure 4-1 - Bacterial growth, palladium (II) and sulphate removal from the medium in the presence of sulphate (1700 mg L^{-1}) and different concentrations of Pd (II): 6 mg L^{-1} (a), 10 mg L^{-1} (b), 18 mg L^{-1} (c) and 26 mg L^{-1} (d). Data are the average of duplicates and error bars indicate the standard deviations of the average values.

Palladium bioremoval from solution was also investigated in the absence of sulphate (Figure 4-2). In solutions containing 6, 10 and 19 mg L^{-1} of palladium (II), the performance of palladium bioremoval was similar to that observed in the presence of sulphate. Approximately 60% of palladium was removed from the solution after 19 days of incubation in the presence of 6 mgL$_{-1}$ and after 6 days in the presence of 10 and 19 mg L^{-1} of palladium. However, in contrast to what was observed in the presence of sulphate, palladium removal was not detected at the highest palladium concentration (27 mg L$_{-}$^{1}). Even though palladium removal has not been affected by the absence of sulphate in three metal concentrations (6, 10 and 18 mg L^{-1}), bacterial growth was affected (Figure 4-2) because an increase of the lag phase was observed for the two lowest metal concentrations. OD$_{600}$ values of 0.25 and 0.18 were observed in the presence of 6 and 10 mg L^{-1} of palladium (II), respectively, after 6 days of incubation. These values were much lower than those observed in the presence of sulphate after the same incubation time (0.65 and 0.60, respectively). However, similar OD$_{600}$ values were reached at the end of the experiment regardless of the presence or absence of sulphate. In addition, no bacterial growth was observed in the presence of 27 mg L^{-1} without sulphate, which is in accordance with the absence of palladium removal for this metal concentration.

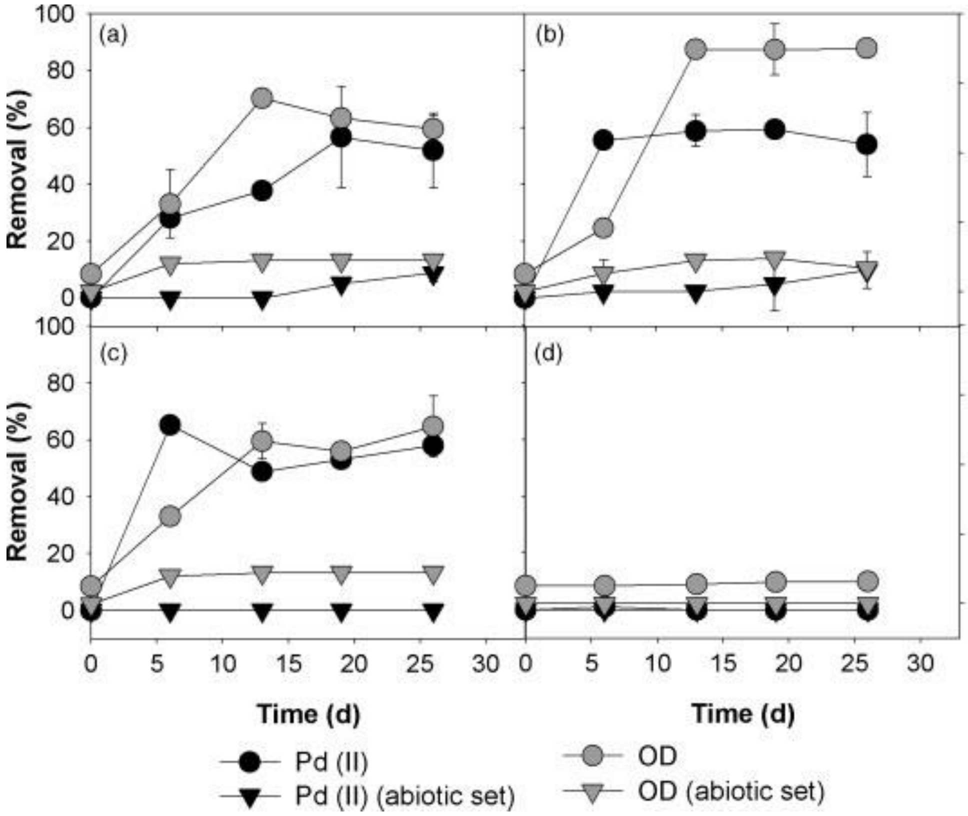

Figure 4-2 - Bacterial growth, palladium (II) removal from the medium in the absence of sulphate and different concentrations of Pd (II): 6 mg L^{-1} (a), 10 mg L^{-1} (b), 19 mg L^{-1} (c) and 27 mg L^{-1} (d). Data are the average of duplicates and error bars indicate the standard deviations of the average values.

3.2. Phylogenetic analysis

The structure of the bacterial communities with the ability for palladium (II) removal from the medium in the presence and in the absence of sulphate was determined in order to identify the bacteria responsible for Pd(II) removal. This identification is particularly relevant taking into account that this consortium was screened among several others, also enriched from environmental samples, which were not successful in terms of Pd(II) resistance and biorecovery capacity. A total of 79 recombinant colonies were recovered and an approximately 1.4 kb fragment of bacterial 16S rRNA gene was amplified and used for RFLP analysis. Twelve RFLP groups were originated from the mixed culture. Five RFLP groups

(Pd+S10, Pd+S40, Pd+S49, Pd+S85 and Pd+S103 as representative clones) originated from the community grown in the presence of sulphate were also originated from the culture grown without sulphate. Furthermore, three and four additional RFLP groups were originated from the community grown in the presence and absence of sulphate, respectively (Pd+S47, Pd+S48, Pd+S52, Pd15, Pd54, Pd58 and Pd72 as representative clones). Phylogenetic analysis of the representative clones allowed identification of the corresponding sequences (Figure 4-3).

Regardless of the presence or absence of sulphate, most of the clone sequences from the bacterial community were closely related to *Clostridium* genus (71% and 74% of clones from the consortia grown without and with sulphate, respectively).

Phylogenetic analysis also showed that 22% and 13% of the clone sequences from the community grown in the presence of palladium (II) without sulphate and with sulphate, respectively, were closely related to *Bacteroides*. Moreover, bacteria affiliated to *Citrobacter braakii* (NR028687) were also present in both communities (7% and 11% of clones from the consortia grown without and with sulphate, respectively). An unidentified bacterium (2%) was also detected in the community grown in the presence of sulphate. Although the original community was composed of SRB (Martins *et al.*, 2010b), this bacterial group was not detected in the presence of 18 mg L^{-1} of Pd(II). These results are in agreement with the loss of ability for sulphate removal. The evolution of the bacterial consortium can probably be explained by the lower resistance to Pd(II) of the SRB initially present in the consortium in comparisonwith *Clostridium* species,which became then the most abundant in both conditions. A similar dynamic was previously observed by Martins and colleagues (2010b), who reported a drastic change in two initial SRB communities during uranium(VI) bioremoval experiments and by Assunção and colleagues (2011), who observed that bacteria affiliated to *Clostridium* and *Citrobacter* species were responsible for sulphate andbromate removal during bromate bioremoval studies. Thus, it is possible to conclude that the bacterial community with the ability to remove Pd(II) from solution is mainly composed of bacteria affiliated to *Clostridium* genus. In fact, these microorganisms have been reported to have excellent tolerance to Pd(II) which can explain their dominance in the community (Chidambaram *et al.*, 2010).

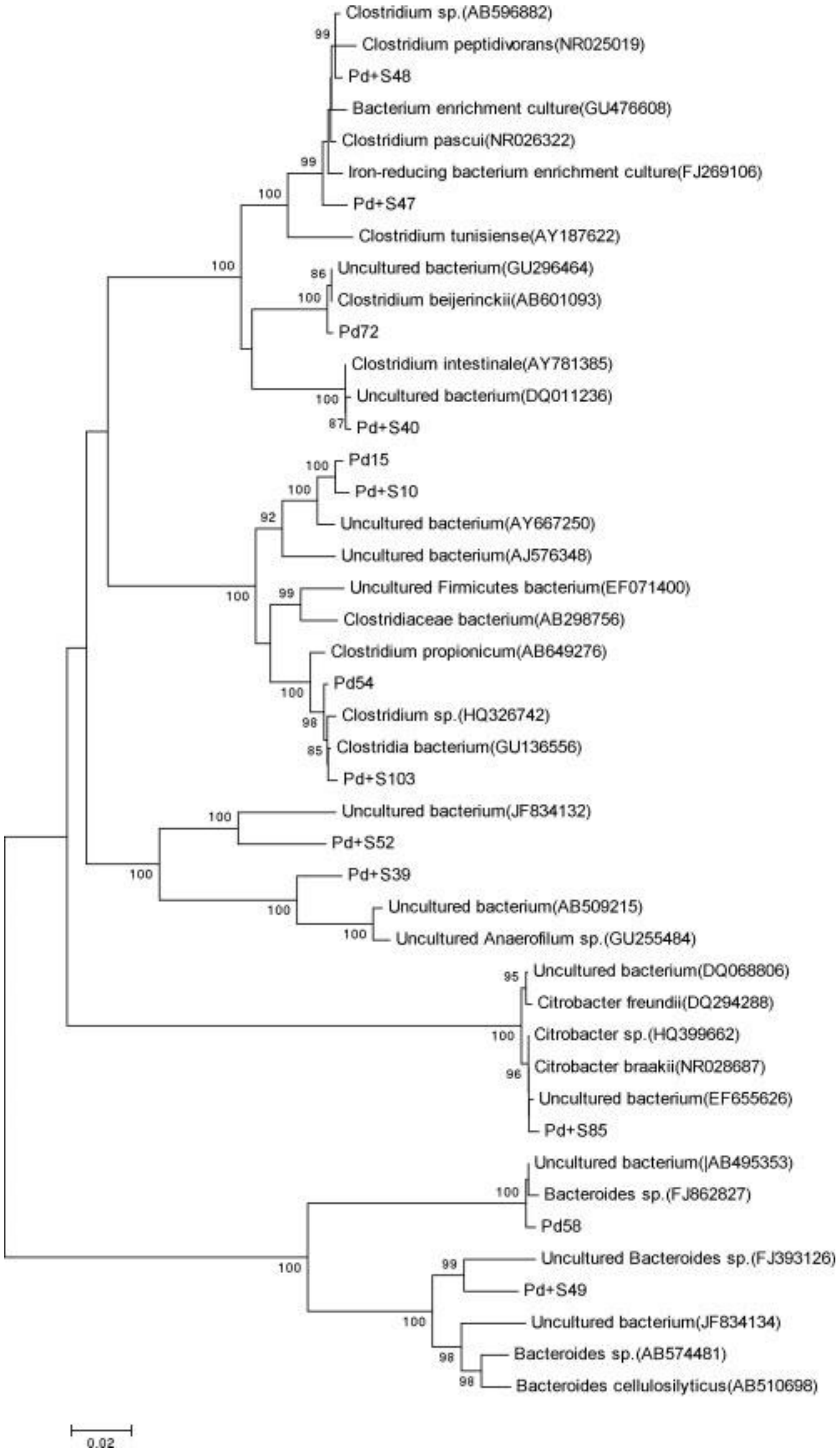
Clostridium sp.(AB596882)
99
Clostridium peptidivorans(NR025019)
Pd+S48
Bacterium enrichment culture(GU476608)
Clostridium pascui(NR026322)
99
Iron-reducing bacterium enrichment culture(FJ269106)
100
Pd+S47
Clostridium tunisiense(AY187622)
100
86
Uncultured bacterium(GU296464)
100
Clostridium beijerinckii(AB601093)
Pd72
Clostridium intestinale(AY781385)
100
Uncultured bacterium(DQ011236)
87
Pd+S40
100
Pd15
100
Pd+S10
92
Uncultured bacterium(AY667250)
Uncultured bacterium(AJ576348)
100
99
Uncultured Firmicutes bacterium(EF071400)
Clostridiaceae bacterium(AB298756)
Clostridium propionicum(AB649276)
100
Pd54
98
Clostridium sp.(HQ326742)
85
Clostridia bacterium(GU136556)
Pd+S103
100
Uncultured bacterium(JF834132)
Pd+S52
100
Pd+S39
100
Uncultured bacterium(AB509215)
100
Uncultured Anaerofilum sp.(GU255484)
95
Uncultured bacterium(DQ068806)
Citrobacter freundii(DQ294288)
100
Citrobacter sp.(HQ399662)
96
Citrobacter braakii(NR028687)
Uncultured bacterium(EF655626)
Pd+S85
Uncultured bacterium(|AB495353)
100
Bacteroides sp.(FJ862827)
Pd58
100
99
Uncultured Bacteroides sp.(FJ393126)
Pd+S49
100
Uncultured bacterium(JF834134)
98
Bacteroides sp.(AB574481)
98
Bacteroides cellulosilyticus(AB510698)
0.02

Figure 4-3 - Phylogenetic tree obtained with 16S rRNA sequences, corresponding to the clones representative of each restriction profile and to the most closely related ones retrieved from BLAST search. Phylogeny was inferred using the Neighborhood-Joining algorithm analysis of aligned 16S rRNA fragments. Bootstrap values are indicated on branches. Access numbers of GenBank sequences are indicated in the figure.

3.3. Mechanism of Pd(II) bioremoval

The mechanism of Pd(II) removal by the bacterial community in the presence and absence of sulphate was also examined. The metal removal could occur through several mechanisms such as biosorption, bioaccumulation or an enzymaticallydriven mechanism, such as metal reduction and consequent precipitation.

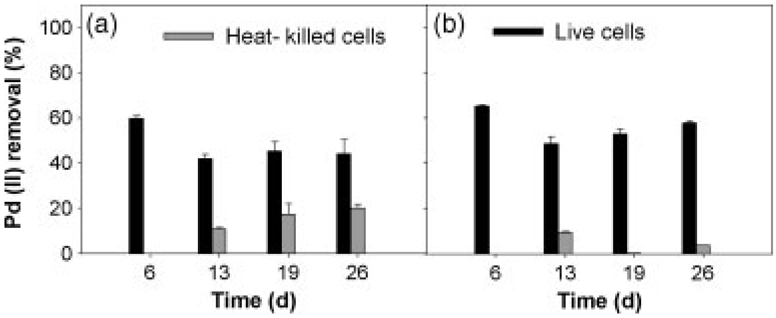

Figure 4-4 - Palladium (II) removal from the medium in the presence of sulphate (a) and in the absence of sulphate (b) and by cells (live and heat-killed) (pH 7.5 ± 0.1). Data are the average of duplicates and error bars indicate the standard deviations of the average values.

Figure 4-4 shows palladium removal by live cells and heat-killed cells in the presence (Figure 4-4(a)) and in the absence of sulphate (Figure 4-4(b)). In the presence of sulphate palladium removal by heat-killed cells reached about 20% after 26 days, while in the medium without sulphate heat-killed cells removed less than 10%. These results suggest that biosorption could be one of the mechanisms involved in Pd(II) removal. However, the values associated with biosorption are much lower than those observed with live cells (59% of metal was removed by cells grown in the presence of Pd(II) and sulphate while 66% was obtained in the medium without sulphate) indicating that the mechanism associated with viable cells could be mainly responsible for palladium (II) removal from solution.

Bioaccumulation and bio-precipitation are the two mechanisms that can be responsible for metal removal by viable cells. Thus, in order to establish the distribution and location of palladium deposits in the cells, thin sections of cells exposed to palladium in the presence and absence of sulphate were viewed by TEM (Figure 4-5).

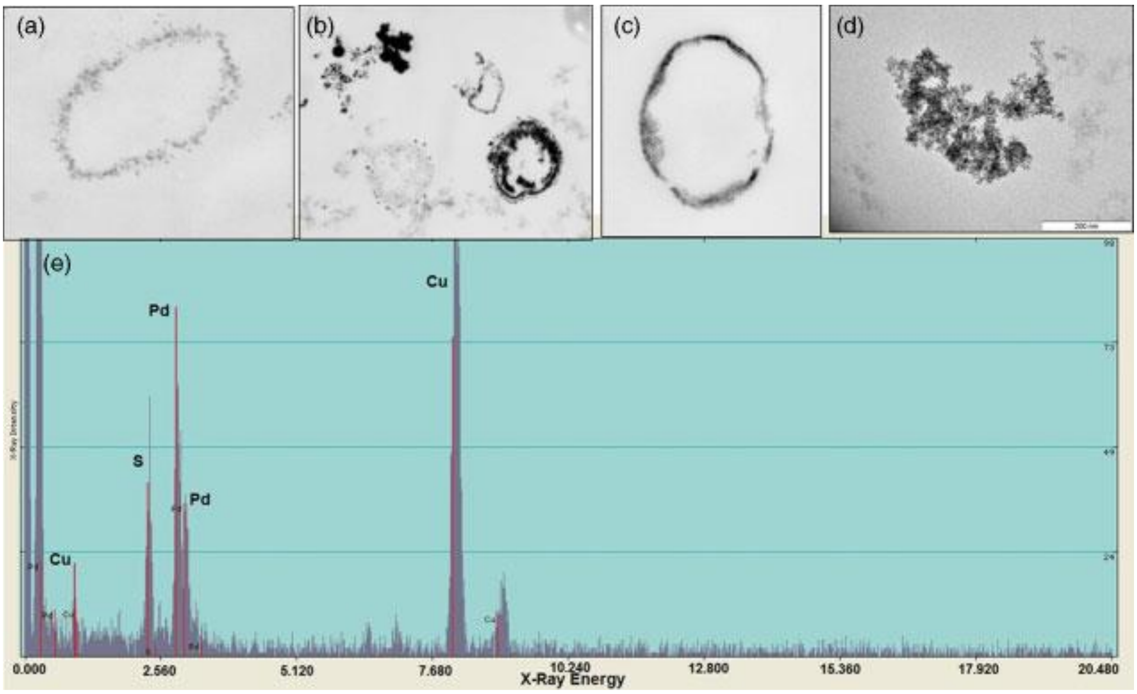

Figure 4-5 - TEM of cells sections without staining (a, b and c) and SEM of extracellular precipitate (d) collected after 26 days of growth with 18 mg L^{-1} Pd (II) plus sulphate. EDS spectrum of the precipitate (e).

According to TEM analysis no differences were observed on the cells grown with palladium in the presence and absence of sulphate. TEM-EDS analysis showed the presence of dense precipitates in the surface of bacterial cells (Figure 4-5(a), (b), (c)), which were mainly composed of palladium (Figure 4-5(e)). Other elements such as sulphur and copper were also detected (Figure 4-5(e)) in the precipitates. The presence of copper could originate from the supporting grid since it was also present in background areas. The presence of sulphur can result from the cells or from the components of the medium. The presence of palladium precipitates in the surface of the bacterial cells suggests that reduction of Pd(II) to Pd(0) followed by precipitation could be the mechanism involved (Rashamuse and Whiteley, 2007; De Corte *et al.*, 2012). In addition, the presence of Pd(0) was also consistent with the black colour of the precipitate formed at the bottom of the glass bottles and by the color change of the solution from pale yellow to brown in the presence of live cells (De Windt *et al.*, 2005).

In contrast, the abiotic controls showed neither a color change nor the appearance of the black precipitates. The X-ray diffraction (XRD) analysis of these biologically generated precipitates showed that they are mainly composed of amorphous material (data not shown). This result can be explained by the high ratio between cells and metal amount.

The occurrence of Pd(II) bioreduction is consistent with the composition of the bacterial community, which is mainly composed of *Clostridium* species. In fact, it has been demonstrated that the H_2 produced by bacteria belonging to the *Clostridium* genus can subsequently reduce Pd(II) to Pd(0) (Chidambaram *et al.*, 2010). Moreover, the reduction of Pd(II) to Pd(0) by H_2 produced by species of *Citrobacter* and *Bacteroides*, that are also present in the community of the present study, has also been reported (Hennebel *et al.*, 2011). Therefore, this pathway maybe involved inpalladium removal by this bacterial community, since the production of H_2 was also detected in our experiments (according to the results obtained by gas-chromatography after 26 days of incubation, the amount of H_2 accumulated in the headspace of the glass bottles was 29.6 mL ± 1.5). Enzymatic reduction of metals, namely Tc(VII), Pu(IV), Se(VI) and U(VI), by members of *Clostridium* genus has also been reported (Bunge *et al.*, 2010) and thus a mechanism of direct enzymatic reduction of Pd(II) to Pd(0) cannot be excluded.

According to the TEM analysis, the external precipitates were nanoparticles with sizes 7–10 nm (Figure 4-5(d)). These results are an important finding considering that the production of Pd(0) nanoparticles by a mixed culture simply obtained from an environmental sample, is reported for the first time. The very low dimension of these Pd(0) particles is generally an additional advantage taking into account their catalytic performance. In addition, their location at the surface of the bacterial cells can also be advantageous making them more easily available for those applications.

4. CONCLUSIONS

The present investigation demonstrates, for the first time, the ability of a Pd(II)-resistant mixed bacterial culture (enriched from a sludge sample from a municipal waste water treatment plant) to recover palladium from an aqueous medium, leading to the formation of Pd(0) nanoparticles. Thus, Pd(0) nanoparticles were obtained in a single step during growth in a minimal medium of the metal resistant community, mainly composed of *Clostridium* species, with economical advantages. This is a clear demonstration of the potentialities of metal-resistant bacterial consortia enriched from environmental samples for the bio-synthesis of metallic nanoparticles.

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CHAPTER 5

Application of urea-agarose gel electrophoresis to select non-redundant 16S rRNAs for taxonomic studies: palladium (II) removal bacteria

ABSTRACT

The 16S ribosomal RNA (rRNA) gene has been the most commonly used sequence to characterize bacterial communities. The classical approach to obtain gene sequences to study bacterial diversity implies cloning amplicons, selecting clones, and Sanger sequencing cloned fragments. A more recent approach is direct sequencing of millions of genes using massive parallel technologies, allowing a large-scale biodiversity analysis of many samples simultaneously. However, currently, this technique is still expensive when applied to few samples; therefore, the classical approach is still used. Recently, we found a community able to remove 50 mg L^{-1} Pd(II). In this work, aiming to identify the bacteria potentially involved in Pd(II) removal, the separation of urea/heat-denatured DNA fragments by urea–agarose gel electrophoresis was applied for the first time to select 16S rRNA-cloned amplicons for taxonomic studies. The major raise in the percentage of bacteria belonging to genus *Clostridium sensu stricto* from undetected to 21 and 41%, respectively, for cultures without, with 5 and 50 mg L^{-1} Pd(II) accompanying Pd(II) removal point to this taxa as a potential key agent for the biorecovery of this metal. Despite sulfate-reducing bacteria were not detected, the hypothesis of Pd(II) removal by activity of these bacteria cannot be ruled out because a slight decrease of sulfate concentration of the medium was verified and the formation of PdS precipitates seems to occur. This work also contributes with knowledge about suitable partial 16S rRNA gene regions for taxonomic studies and shows that unidirectional sequencing is enough when Sanger sequencing cloned 16S rRNA genes for taxonomic studies to genus level.

Keywords: Palladium bioremoval, 16S rRNA, Bacterial communities, Taxonomic classification

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1. INTRODUCTION

Molecular biology techniques have been used during the last years to characterize bacterial communities. In 1977 Carl Woese, George Fox, and others proposed the use of ribosomal RNA (rRNA) to determine relationships covering the entire spectrum of extant living systems (Woese and Fox 1977, Fox *et al.*, 1977). Subsequently, rRNA genes have been the most commonly used sequences in phylogenetic, taxonomic, and population studies. Thus, the massive work carried out in the last 30 years on DNA sequencing has led to the accumulation of information on these sequences for a large number of organisms.

Although different bioinformatics tools have been developed to analyze the sequences, the principle of the process is unique and can be summarized as follows: highly conserved regions supporting the constancy of the rRNA genes complex secondary structure and function are used to ensure positional homology in sequence alignments, which in their turn are used, taking advantage of the interspersed hypervariable regions, for the attribution of taxonomic classifications and for the construction of phylogenetic trees supporting evolutionary hypothesis.

The use of primers for the conserved domains flanking the hypervariable regions enables robust specific PCR amplifications of target sequences. Thus, the PCR became the preferred approach to obtain rRNA gene sequences to analyze natural or cultured populations and, relying on the objectives of the studies, the different strategies are mainly distinguished by the target genes and by the primers used to amplify them.

In general, for bacteria and archaea, the 16S rRNA gene encoding the 16S rRNA small subunit has been the most important target sequence for these types of studies (Yarza *et al.*, 2008, 2014). In this case, PCR universal primers for the 16S rRNA gene are generally used when the aim is to characterize all population (e.g., Weisburg *et al.*, 1991; Baker *et al.*, 2003). When the objective is to characterize just a taxonomic group of organisms, specific primers for that group have to be designed. For example, specific primers for sulfate-reducing bacterium (SRB) 16S rRNA genes have already been designed and used for phylogenetic, taxonomic, and population studies (e.g., Devereux *et al.*, 1989; Castro *et al.*, 2000; Daly *et al.*, 2000; Karr *et al.*, 2005). Another possibility is the use of genes that are only present in the group of organisms to be studied. For example, in the case of SRB, the dsr gene, encoding the enzyme dissimilatory sulfite reductase (DSR) responsible for the central energy conserving step of sulfate respiration (Odom and Peck, 1984), has proven to be a good alternative (e.g., Wagner *et al.*, 1998; Karr *et al.*, 2005).

The most commonly used strategy in the past (which therefore can be considered a classical approach) to obtain a number of DNA sequences to study bacterial diversity implies the following several steps: (1) PCR amplification of target genes (usually the 16S rRNA gene) or parts of them in a sample, (2) cloning the amplicons by insertion in a vector and transformation into Escherichia coli, (3) selecting a number of transformed colonies, (4) multiplying the number of copies of each cloned amplicon by growing E. coli pure cultures and purifying the plasmids or by direct PCR and purifying the amplicons, and (5) finally sequencing the cloned fragments through the Sanger method. The negative aspect of this strategy is the time and the cost associated to it when the objective is high-depth sampling to detect rare taxa in complex natural or cultured populations.

More recently, with the advent of massive parallel sequencing technologies, direct sequencing of millions of 16S rRNA genes became feasible in a short time (Liu *et al.*, 2008; Armougom and Raoult, 2009), allowing large-scale biodiversity analysis capable of revealing rare taxa in complex communities (e.g., Sogin *et al.*, 2006; Wilhelm *et al.*, 2014). Moreover, massive parallel DNA sequencing with barcoding techniques allows simultaneous deep sequencing of many samples per run (e.g., Hamady *et al.*, 2008), being in that case the cost per sample relatively low. Nevertheless, currently, this technique is still expensive when applied to just one or few samples.

Thus, the classical approach is still applied in studies aiming to characterize the main taxa present in only one or in few bacterial communities and when the identification of rare taxa is not important. In these cases, usually, a DNA fingerprinting analysis of 16S rRNA gene-cloned amplicons is carried out to identify different clones, avoiding sequencing similar ones and therefore reducing the costs in DNA sequencing. The fingerprinting methods most applied for the selection of non-redundant cloned amplicons to characterize bacterial communities are as follows: denaturing- or temperature gradient gel electrophoresis (DGGE or TGGE) (Fischer and Lerman, 1979; Rosenbaum and Riesner, 1987), single-stranded conformation polymorphism (SSCP) (Orita *et al.*, 1989), and amplified ribosomal DNA restriction analysis (ARDRA) (Dijkshoorn *et al.*, 1998). For example, Karr and colleagues (2005) to explore the biodiversity of SRB in Lake Fryxell located in Antarctica have amplified by PCR the dsr genes on water and sediments from this extreme environment and have separated the polymorphic products to be cloned and sequenced by DGGE.

Nevertheless, all these fingerprinting methods referred above have some drawbacks. For the DGGE analysis, the gels are made of acrylamide, which has carcinogenic effects; making gels with denaturing gradients is difficult and requires a wide experience in the preparation of acrylamide gels; the amount of eluted amplicon in each gel excised band is low; and thus, usually, a PCR reamplification is necessary before cloning the fragments to be sequenced, which increases the number of amplification errors. For the TGGE, the drawbacks are similar to those in DGGE and though the preparation of gels is relatively simpler, a special electrophoresis system allowing temperature gradients is necessary. The SSCP method also implies simple acrylamide gels; however, the sensitivity for polymorphisms detection is applicable only in products with sizes up to about 300 bp. Regarding the selection of clones by ARDRA, the principal disadvantage is that the detection of polymorphisms does not cover the entire length of the nucleotide sequences, but is limited to the regions recognized by the restriction enzymes used.

Palladium, a platinum group metal (PGM), is a metal with high economic value due to the limited global resources and high demand, mainly due to its use in catalytic processes (Deplanche *et al.*, 2014). For these reasons, it is nowadays very important to recover and reuse palladium. Some chemical technologies, as electrochemical recover and liquid-liquid extraction, have been mentioned as having ability to treat effluents containing PGM. However, the chemical processes frequently present several disadvantages, such as generation of other effluents/wastes/pollutants, high costs, and inefficiency in the recovery of PGM from diluted solutions.

During the last years, several biological processes to recover PGM have been tested due to their economic and environmental attractiveness. Some pure bacteria cultures have shown ability to remove several metals from aqueous solution. For example, *Desulfovibrio desulfuricans* has been reported as Pd(II) reducer at the expense of H_2 (Yong *et al.*, 2002; Baxter-Plant *et al.*, 2003) and Vargas and colleagues (2004) demonstrated that this sulfate-reducing bacterium (SRB) has more potential for Pd and Pt biosorption than *Desulfovibrio fructosivorans* and *Desulfovibrio vulgaris*. *Shewanella oneidensis* was also reported with biosorption ability and subsequent Pd(II) bioreduction with H_2 (De

Windt *et al.*, 2005). Cells of Bacillus sphaericus also proved to accumulate high amounts of toxic metals, including Pd (Pollmann *et al.*, 2006). More recently, De Corte and colleagues (2012) discussed the different bio-Pd precipitating microorganisms in which they included all the bacteria mentioned above as well as *Citrobacter braakii* (Hennebel *et al.*, 2011) and *Clostridium pasteurianum* (Chidambaram *et al.*, 2010).

Another reason highlighting the importance of studies that might contribute for a better understanding of the cellular responses to PGM is that platinum-based complexes exhibit activity against tumor cells and thus have been extensively applied in chemotherapy. The bond between a platinum compound and DNA has consequences on the structure of protein-DNA complexes and can affect replication and transcription of chromatin, triggering pathways that lead to cell death, a desired consequence in cancer treatment (Jung and Lipapard 2007). Thus, knowledge about uptake or efflux active systems regulating platinum-based complexes homeostasis can become extremely important as it may lead to the development of selective forms of attacking cancer cells. The same goes for palladium-based complexes, one of the alternatives to platinum-based drugs that have shown considerable promise when researchers start to look for alternatives due to side effects associated with cisplatin administration and limited applicability arising from specificity to certain cancer cells (Kapdi and Fairlamb, 2014). For many years, it had been taken for granted that cisplatin enters cells largely by passive diffusion; however, evidence for a role of different active transporters (e.g., copper-transporting P-type ATPases (ATP7A and ATP7B) and copper uptake protein Ctr1) in the uptake and efflux of cisplatin and other platinum compounds has been confirmed, indicating that platinum drugs cross cell membranes through multiple routes (Jung and Lipapard, 2007). Many of these mechanisms have been discovered due to the observation of a different level of resistance or susceptibility to the metal compound in model species. For example, this was the case when a direct connection between the presence of a copper transporter and cisplatin uptake was discovered in a mutagenesis experiment in yeast (Ishida *et al.*, 2002). Therefore, the identification of bacteria resistant to palladium may be the starting point for the discovery of novel such mechanisms.

Our research group reported for the first time a Pd(II)-resistant mixed bacterial culture enriched from a sludge sample from a municipal wastewater treatment plant (WWTP) able to remove 18 mg L^{-1} of Pd(II) from an aqueous medium and the phylogenetic analyses showed that this culture was mainly composed by *Clostridium* species (Martins *et al.*, 2013). Recently, we found a new bacteria community, also enriched from a WWTP sludge sample, resistant and able to remove even higher concentrations of Pd(II) up to 50 mg L^{-1}.

In this work, aiming to identify bacteria potentially involved in Pd(II) removal and to understand the evolution of the bacterial consortium when the Pd(II) concentration is increased, the new consortia was grown in the absence of Pd(II) and in the presence of 5 and 50 mg L^{-1} of Pd(II). Then, having only three samples, and because the aim was to identify the main groups of bacteria that constitute the bulk of the communities and not rare taxa, we characterized the bacteria consortia based on 16S rRNA gene sequences selected following the above considered classical approach. To that purpose, a new simple and sensitive method to detect polymorphisms based on urea–agarose gel electrophoresis (Hegedüs *et al.*, 2009) was tested and applied to select representative cloned amplicons for Sanger sequencing in order to taxonomically classify their origin and thus characterize the major bacterial groups in the consortia. Moreover, we compared the taxonomic classifications retrieved with sequences from one half side of the 16S rRNA gene with classifications obtained with sequences from the other half side of the gene obtained by sequencing cloned amplicons with two universal plasmid primers flanking the cloning site, to study the feasibility of using only one of the primers to reduce sequencing costs.

2. MATERIAL AND METHODS

2.1. Palladium (II) bioremoval

2.1.1. Biological reactors

All assays and the initial bacterial community enrichment were performed in batch reactors using glass bottles (120 mL) containing 100 mL of nutrient medium with pH adjusted to 7.5 ± 0.2 under anaerobic conditions at room temperature (21 ± 1°C). In order to achieve the anaerobic conditions before inoculation, the medium was purged with nitrogen gas, and after inoculation, about 10 mL of liquid paraffin was added. The bottles were sealed with butyl rubber stoppers and aluminum seals and incubated at room temperature. Growth media and material used in batch experiments were sterilized by autoclaving.

2.1.2. Source and enrichment of the bacterial community

The bacterial consortium used in the present study was enriched from a sludge sample from a wastewater treatment plant, located in Lagos, in southern Portugal.

The medium used for this enrichment was Postgate E (Postgate, 1984) without agar and supplemented with resazurin as redox indicator (0.03 g L^{-1}). Postgate E is a medium developed for SRB, a group of microorganisms known to be able to reduce sulfate to sulfide, thus removing metals from aqueous media as insoluble metal sulfides.

2.1.3. Experimental frame

The medium used in Pd(II) removal experiments was based on Postgate C (Postgate, 1984), which is also a growth medium for SRB, but modified in order to maintain Pd(II) soluble. First, a culture was grown in this medium but without Pd(II) to acclimatize the bacteria. This culture was prepared using 10 % (v/v) inoculum harvested from the enrichment by centrifugation at 2500×g for 10 min and washed with modified Postgate C medium without palladium.

The modified Postgate C medium contained 0.5 g L^{-1} KH_2PO_4, 1 g L^{-1} NH_4Cl, 4.5 g L^{-1} Na_2SO_4, 0.06 g L^{-1} $CaCl_2.6H_2O$, 1 g L^{-1} yeast extract, 0.0072 g L^{-1} $FeSO_4.7H_2O$, 0.06 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.3 g L^{-1} Tri-sodium citrate dehydrate, 0.1 g L^{-1} NaCl, and 6.0 g L^{-1} sodium lactate (the difference to the original Postgate C is the addition of NaCl and a lower content (0.0072 g L^{-1}) of $FeSO_4.7H_2O$).

The first experiment with Pd(II) was performed adding 10 % (v/v) inoculum of the previously acclimatized culture to the modified Postgate C medium supplemented with 5 mg L^{-1} of Pd(II), as Pd(II) nitrate. The culture grown in the presence of 5 mg L^{-1} of Pd(II) was then used as inoculum (10% (v/v)) for another culture in the same conditions but containing a higher concentration of Pd(II) 50 mg L^{-1}.

In order to evaluate the relation between Pd(II) removal and the bacterial growth, abiotic assays were made in exactly the same conditions as the cultures with Pd(II), but without bacterial inoculum.

The biotic assays (with bacterial inocula) in growth media with Pd(II) were performed with four replicates. The biotic assay without Pd(II) and all abiotic assays were carried out with two replicates.

2.1.4. Analytical methods

The optical density (OD_{600}) was determined weekly in order to monitor the bacterial growth. In addition, aiming to monitor an eventual growth of SRB, the oxidation-reduction potential (Eh) and the sulfate concentration were also measured weekly. The pH was monitored due to its importance as a limiting factor, and palladium concentration was determined to evaluate its removal from the growth media.

The samples from batch cultures were collected using a sterile syringe and OD_{600} was immediately measured in each sample. Then, the samples were centrifuged at 2500 ×g for 5 min and the supernatant was used for the remaining analysis. Redox potential (Eh) and pH were determined using a pH/E meter (GLP 21, Crison, Barcelona, Spain). Sulfate concentration was quantified by UV-visible spectrophotometry (DR2800 spectrometer, Hach-Lange, Düsseldorf, Germany) using the method of SulfaVer®4 from Hach-Lange (Düsseldorf, Germany). Palladium concentration in the media was determined by flame atomic absorption spectroscopy (Flame-AAS) using an Analyticjena (Jena, Germany) novAA 350 model spectrometer.

The precipitates were obtained collecting the samples by centrifugation at 2500×g for 10 min; the pellet was washed with ethanol 70% (stirred and centrifuged 2500×g for 20 min, three times) and then dried in vacuum (Binder, VDL) at 37°C ± 1°C. In order to confirm the particles size, morphology, and position in relation to cells, a transmission electron microscopy (TEM) analysis was made using a Hitachi (Tokyo, Japan), H8100 model, with a LaB6 filament. This analysis was coupled to an energy dispersive X-ray spectrometer (EDS) for light elements, NORAN System (Thermo Scientific, Waltham, USA), aiming to determine the elemental composition of the precipitates.

2.2. Molecular characterization of bacterial communities

2.2.1. DNA extraction, PCR amplification, and cloning 16S rRNA gene amplicons

At the end of the experiment, 5 mL samples from the bacterial cultures grown in the absence of palladium as well as the bacterial communities resistant and with ability to remove 5 mg L^{-1} and 50 mg L^{-1} of Pd(II) were centrifuged at 2500×g for 10 min to collect cells and their DNA was extracted as described by Martins and colleagues (2009).

The full-length 16S rRNA gene amplification was carried out using the primers 8F (5′- AGA GTT TGATCC TGG CTC AG -3′) and 1492R (5′- GGT TAC CTT GTTACG ACT T-3′) (Suzuki *et al.*, 2003) acquired from NZYTech (Lisbon, Portugal). For PCR amplifications, the following mixture was used: 31.75 µL of sterilized Mili-Q water, 1 µL of each primer (10 pmol/µL), 1 µL of dNTP's (10 mM), 4 µL of $MgCl_2$ (25 mM), 10 µL of 5× GoTaq® buffer (Promega, Madison, USA), 0.25 µL of GoTaq® DNA polymerase (Promega, Madison, USA), and 1 µL of DNA (5 to 50 ng/µL). The PCR amplification was accomplished in a thermocycler (2720 Thermal Cycler, Applied Biosystems, Foster City, USA) using an initial denaturation step of 94°C for 3 min, followed by 35 cycles of 94°C for 1 min, 57°C for 1 min and 72°C for 2 min, and a final step of 5 min at 72°C.

The PCR products were analyzed by electrophoresis in 1% (w/v) agarose gels in 1× TAE buffer (AMRESCO, Solon, USA). The DNA band with the desired size, around 1.4 Kb, was excised and the purification was carried out with E.Z.N.A. TM Gel Extraction Kit (Omega, Norcross, USA). For cloning, the purified products were ligated into the vector pGEM®-T Easy (Promega, Madison, USA) with T4 ligase enzyme and transformed into *E. coli* XL-1 Blue competent host cells, according to the manufacturer's instructions.

Thirty-two transformed (white) colonies were randomly selected from cultures without Pd, with 5 mg L^{-1} Pd(II) and with 50 mg L^{-1} Pd(II) for subsequent taxonomic classifications and consortia characterization. In order to rapidly multiply and isolate the cloned products, a PCR with vector specific primers SP6 and T7 was carried out directly from bacteria by touching the colony with a pipette tip and submerging it in the reaction mixture. The PCR was carried out in a thermocycler with the following conditions: denaturation of 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, and a final step of 5 min at 72°C.

2.2.2. Screening of cloned amplicons by urea–agarose gel electrophoresis

Urea–agarose gel electrophoresis - The procedure to prepare and run the samples in urea–agarose gel electrophoresis was adapted from Hegedüs and colleagues (2009) with minor modifications. Before loaded on the gels, samples were urea-/heat-denatured. For that purpose, 2.5 µL of the amplified DNA samples were added to 12.5 µL of urea-loading buffer [0.5 mg/ml bromephenol blue (Merck, Kenilworth, USA), 8 M urea, 9% glycerol and 1 mM Tris pH 8]; the mixtures were heated at 90°C for 5 min and immediately placed on ice. For the urea–agarose gel electrophoresis, gels of 1.2% (w/v) agarose were prepared and run in 1× TAE buffer supplemented with 1 M urea. DNA was stained by adding 50 µL L^{-1} of GreenSafe Premium (NZYTech, Lisbon Portugal) to gels and the electrophoresis was accomplished with 110 V in an electrophoretic tank at 4°C for 4 h.

Validation of similar amplicons selection - The efficiency of running urea/heat-denatured 16S rRNA amplicons on urea–agarose gels for the identification of similar 16S rRNA sequences was evaluated by comparing several cloned fragments exhibiting equal gel migration patterns for their (1) identity similarities calculated with aligned sequences trimmed for quality and cropped to the same size, (2) position on a phylogenetic tree, and (3) taxonomic classifications.

2.2.3. Amplicons purification and sequencing

PCR products were precipitated with absolute ethanol, washed with 70% (v/v) ethanol and resuspended in Mili-Q water. The DNA was quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, USA) and

Sanger sequenced with the primers SP6 and/or T7 using a capillary electrophoresis sequencing system (Genetic Analyzer, Model 3130xl, Applied Biosystems, Foster City, USA). Based on the analysis of the chromatograms, the obtained sequences were cropped to eliminate the beginning and the ending regions with doubtful profiles using the program BioEdit Sequence Alignment Editor (Hall, 1999).

The presence of putative chimera in the amplified gene sequences was first verified by us using the web tool DECIPHER (http://decipher.cee.wisc.edu/FindChimeras.html) and then by the NCBI GenBank Submissions Staff.

2.2.4. 16S rRNA based taxonomy

Classification method - The RDP Naive Bayesian rRNA Classifier, which showed to be accurate down to the genus level for 400 bp partial 16S rRNA sequences (Wang *et al.*, 2007), was used for taxonomic classifications. Version 2.10 was used online at https://rdp.cme.msu.edu/classifier/classifier.jsp with default settings. The RDP 16S rRNA training set 10 was used, and gene copy number adjustments were based on copy number data rrnDBv4.2.2 provided by rrnDB website http://rrndb.umms.med.umich.edu/ (Stoddard *et al.*, 2014).

Gene regions - Ten cloned 16S rRNA gene amplicons from the consortium grown with 5 mg L^{-1} Pd(II) were sequenced with SP6 and T7 vector universal primers flanking the cloning site, and taxonomic classifications obtained independently with both gene parts were compared.

2.2.5. *Phylogenetic tree construction*

The phylogenetic tree was constructed with the 16S rRNA partial gene sequences obtained for the consortium grown with 5 mg L^{-1} Pd(II) (several exhibiting equal gel migration patterns) and a set of 57 16S rRNA reference gene sequences.

The set of 57 reference sequences was previously chosen from a database of 7081 complete 16S rRNA sequences (DataSet S2) identified by Větrovský and Baldrian (2013) in publicly available complete bacterial genomes. The selection was carried out by local BLAST search with BlastStaion (version 2.0) to characterize a sludge bacterial community enriched from a wastewater treatment plant (WWTP) located in Algarve, Portugal (unpublished data), such as the initial consortium used in the present work.

After trimming low-quality ends, the partial sequences of cloned genes were oriented towards the 16S rRNA open reading frame (ORF) and aligned with the 57 reference sequences using the CLUSTALW Multiple Sequence Alignment tool available online at http://www.genome.jp/tools/clustalw/. The accuracy of these alignments was confirmed by careful observation using the program BioEdit (Hall, 1999).

The program MEGA, version 6.06, (Tamura *et al.*, 2013) was used to choose the best substitution model to infer phylogenetic trees for the aligned sequences, with the maximum likelihood statistical method and the neighbor-joining (NJ) tree algorithm.

The phylogenetic trees were built using the identified best model and the BIONJ algorithm, an improved version of the NJ (Gascuel, 1997), with PhyML3.0 algorithms, methods, and utilities (Guindon *et al.*, 2010) in the ATGC South of France bioinformatics platform (http://www.atgc-montpellier.fr/phyml/). Reliability for internal branching was assessed using 100 bootstrap replicates.

2.3. Nucleotide sequence accession numbers

Sequences for cloned 16S rRNA gene amplicons obtained in this study have been deposited in NCBI and have GenBank accession numbers KT452863 to KT452896.

3. RESULTS

3.1. Palladium (II) bioremoval

3.1.1. Bacterial growth

No significant pH variation was observed in both abiotic and biotic assays with values very close to neutral (7.0 to 7.3) and, as shown in Figs. S1, S2, and S3 in the Appendix 1, all the bacteria communities grown in the absence of palladium and in the presence of 5 and 50 mg L^{-1} of Pd(II) showed fast growth, reaching OD_{600} values above 0.6 after 6 days, and stayed active during the experiment, with even higher OD_{600} values.

Another evidence of bacterial growth and activity was that despite during the experiment, the redox potential (Eh) decreased in all assays, the magnitude of this decays was clearly different when comparing biotic and abiotic assays. In the biotic assays, the Eh values decreased drastically from +99, +101, and +199 mV in the beginning of experiments to −189 ± 10.6, −208 ± 4.0, and −317 ± 6.7 mV at the end, respectively, for cultures without Pd(II), with 5 mg L^{-1} Pd(II) and with 50 mg L^{-1} Pd(II). In the abiotic controls prepared with media containing 5 mg L^{-1} Pd(II) and 50 mg L^{-1} Pd(II), the Eh varied, respectively, from the initial +101 and +199 to −29 ± 1.4 and −29 ± 11.3 mV at the end. This decrease of Eh in the abiotic assays is due to the presence of sodium citrate in the culture medium. This reducing agent is used because the chosen culture medium is appropriate for SRB, and one of the major prerequisites for cultivating these bacteria is that the Eh must be negative. Thus, the much more pronounced declines of Eh in the biotic tests indicate biological production of one or more reducing agents. That could be an indication H_2S production by SRB activity; however, despite the use of a growth medium which composition was based on a medium for SRB, the sulfate was not substantially consumed (Figs. S1, S2, and S3 in the Appendix 1), suggesting that SRB were not present or, more probably, were in a minority. The highest Eh decrease obtained for the consortium grown with 50 mg L^{-1} of Pd(II) can probably be due to a higher Pd(II) removal.

3.1.2. Palladium (II) removal

Regarding Pd(II) removal, the community grown with 5 mg L^{-1} of Pd(II) showed ability to remove 91% of this metal after 21 days of incubation (Fig. S2 in the Appendix 1) while bacteria grown in the presence of 50 mg L^{-1} of Pd(II) showed ability to remove 98% of Pd(II) after 28 days (Fig. S3 in the Appendix 1). These good performances for Pd(II) removal can be attributed to the growth of the bacterial communities since in the abiotic (negative) controls the initial Pd(II) concentrations remained in the medium during all the incubation time (Figs. S4 and S5 in the Appendix 1). The decrease of Pd(II) concentration in the biotic assays was accompanied by formation of dark-colored precipitates while in the abiotic controls, precipitate's formation was not detected.

3.1.3. Precipitates analysis

TEM analysis allowed concluding that the particles composing the precipitates are nanoparticles with sizes between 12 and 32 nm distributed in agglomerates along the bacterial cells and also individualized and presenting a spherical morphology (Fig. S6 a–c in the Appendix 1).

The coupled EDS analysis detected the Pd and S elements in the particles, which is a strong indication that the particles are effectively palladium sulfide (PdS) (Fig. S6 d in the Appendix 1). Peaks corresponding to the carbon and copper elements were also detected in the EDS spectrum (Fig. S6 d in the Appendix 1). However, these elements are components of the supporting grid and the respective peaks are detected in background areas.

3.2. Molecular characterization of bacterial communities

3.2.1. PCR amplification and cloning 16S rRNA gene amplicons

The agarose gel electrophoresis of PCR products amplified using the 16S rRNA universal primers 8F and 1492R with DNA extracted from all bacterial communities revealed major bright bands with the expected size (approximately 1.4 Kb), indicating full-length amplifications of the gene target.

Afterwards, amplified genes were successfully ligated to plasmid vectors which were used to transform E. coli and 32 recombinant (white) colonies from each community were selected for further PCR amplifications. True positives (generating products of approximately 1.6 Kb) were confirmed by agarose gel electrophoresis for 27, 30, and 22 colonies, respectively, from the bacterial communities grown without, with 5 and with 50 mg L^{-1} of Pd(II).

3.2.2. Screening of cloned amplicons by urea–agarose gel electrophoresis

Aiming to avoid sequencing all cloned products selected for each consortium, the simple and sensitive DNA fingerprinting analysis method based on urea–agarose gel electrophoresis described by Hegedüs and colleagues (2009) was tested and applied to identify similar cloned amplicons.

Urea–agarose gel electrophoresis - The analysis of the cloned 16S rRNA amplicons by urea–agarose gel electrophoresis DNA fingerprinting allowed to group clones according to their migration patterns. After heat denaturation in the presence of 8 M urea, the two strands of the cloned 16S rRNA gene fragments migrated differently in the 1 M urea containing agarose gels in the size range of 0.8 to 1.2 Kb (Figure 5-1).

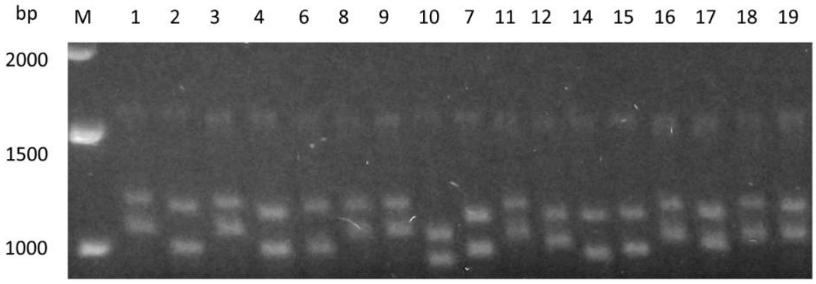

Figure 5-1 - Migration patterns of 17 16S rRNA cloned amplicons from the culture grown with 5 mg L^{-1} Pd(II) after urea-/heat-denaturation and urea–agarose gel electrophoresis at 110 V during 4 h at 4°C. DNA was stained by adding 50 µL L^{-1} of GreenSafe Premium (NZYTech) to gels and exposure to UV light. Gel pattern type 1: 2, 6, 14, 15; gel pattern type 2: 1, 3, 8, 9, 11, 16, 18, 19; gel pattern type 3: 4, 7, 17; gel pattern type 4: 12; gel pattern type 5: 10.

The different gel migration patterns identified among clones of each bacterial community and the numbers of clones exhibiting those patterns are listed in Table 5-1.

Table 5-1 - Different 16S rRNA gene urea-agarose gel electrophoresis migration (fingerprinting) patterns and numbers of clones exhibiting them for each consortium

Consortium	Nº of white colonies tested	Nº of true positives	Gel pattern type	Nº of clones
Grown without Pd(II)	32	27	noPd-gpt-1	8
			noPd-gpt-2	5
			noPd-gpt-3	7
			noPd-gpt-4	1
			noPd-gpt-5	2
			noPd-gpt-6	1
			noPd-gpt-7	1
			noPd-gpt-8	2
Grown with 5 mg L^{-1} Pd(II)	32	30	5Pd-gpt-1	7
			5Pd-gpt-2	10
			5Pd-gpt-3	11
			5Pd-gpt-4	1
			5Pd-gpt-5	1
Grown with 50 mg L^{-1} Pd(II)	32	22	50Pd-gpt-1	3
			50Pd-gpt-2	5
			50Pd-gpt-3	8
			50Pd-gpt-4	4
			50Pd-gpt-5	1
			50Pd-gpt-6	1

Validation of similar amplicons selection - Eighteen cloned 16S rRNA amplicons from the consortium grown with 5 mg L^{-1} Pd(II) were sequenced, and the sequences from those exhibiting identical urea–agarose gel electrophoresis migration patterns were used to confirm the efficiency of running such type of electrophoresis for the identification of similar 16S rRNA genes. The efficiency was evaluated by comparing (1) identity similarities calculated with aligned sequences trimmed for quality and cropped to the same size, (2) position on a phylogenetic tree, and (3) taxonomic classifications.

The high identity similarities calculated between sequences of amplicons with the same gel pattern type was the first sign to confirm this efficiency. Fourteen sequences from eight cloned amplicons with gel pattern type 5Pd-gpt-2 revealed very high identity similarities (between 99.1 and 100%). Four sequences from three cloned amplicons with gel pattern type 5Pd-gpt-3 also showed very high similarities (98.9 to 100%). Six sequences from four cloned amplicons with gel pattern type 5Pd-gpt-1 still showed high similarities but at a lower level (78.8 to 98.9%). The taxonomic classifications retrieved with the RDP classifier for sequences of the 16S rRNA amplicons with the same gel migration pattern are in accordance with the high similarities revealed within them. That is, all urea-/heat-denatured 16S rRNA gene amplicons separated by urea–agarose gel electrophoresis exhibiting an identical migration pattern were classified in the same genus or in related genus belonging to closely related families of the same order (Table S1 in the Appendix 1). All sequences from amplicons with gel pattern type 5Pd-gpt-2 were classified to genus *Arcobacter* and those with gel pattern type 5Pd-gpt-3 were classified to genus Parabacteroides with high estimated confidences (>95%). The two amplicons with pattern type 5Pd-gpt-1 (excluding the other two that were deciphered to be chimerical) were classified in the order *Clostridiales*, with 100% confidence: one in the family *Clostridiaceae* 1, genus *Clostridium sensu stricto*, with 100% confidence, and the other in the family *Clostridiaceae* 3, genus *Clostridiisalibacter*, with 43 and 32% confidence, respectively. With an estimate of confidence of 100%, the classification as *Clostridium sensu stricto* is more likely the correct one.

The positions of the cloned amplicons on the phylogenetic tree also confirm the reliability of identifying similar 16S rRNA amplicons by urea–agarose gel electrophoresis. Each group of 16S rRNA amplicon sequences with a particular gel migration pattern stayed in a cluster noticeably separated from the other groups of 16S rRNA sequences with different gel migration patterns (Fig. S7 in the Appendix 1). In addition, the grouping of sequences obtained in this work with the reference sequences confirms the taxonomic classification obtained by RDP classifier. Fourteen sequences from 8 amplicons exhibiting gel migration pattern type 5Pd-gpt-2 gathered in a cluster, with 95 bootstrap strength, together with sequences from species belonging to the order *Campylobacterales*, family *Campylobacteraceae* (Fig. S7 in the Appendix 1 - names in purple). Six sequences from 4 amplicons with the gel migration pattern type 5Pd-gpt-1, including 2 deciphered to be chimeric, stayed in a cluster with a bootstrap value of 40, together with sequences from specimens of order *Clostridiales*, families *Clostridiaceae* and *Eubacteriaceae* (Fig. S7 in the Appendix 1 - names in orange). Five sequences of 4 amplicons with the gel migration pattern type 5Pd-gpt-3 grouped, with a bootstrap robustness of 70, with species from order *Bacteroidales*, family *Porphyromonadaceae* (Fig. S7 in the Appendix 1 - names in green).

3.2.3. 16S rRNA-based taxonomy

There are two major aspects of bacterial community studies using 16S rRNA gene sequences determining the extent to which these studies are effective: the gene regions that are used and the method used for taxonomic classification.

Gene regions - The sequences obtained with SP6 and T7 primers (from both sides of the vector cloning site) covered different hypervariable regions of the 16S rRNA gene according to its orientations in the cloning vector, with sizes between 477 and 934 bp.

Looking to the 20 sequences of the 10 cloned 16S rRNA amplicons sequenced with both primers SP6 and T7 (clones 5Pd-c-2, 3, 9, 11, 12, 14, 16, 18, 19, and 20), it can be seen that (1) when sequencing was orientated from the beginning to the end of the gene, all 10 sequences cover completely the hypervariable regions V1, V2, and V3 and partially regions V4 and (2) when sequencing was orientated towards the other side, all 10 sequences cover hypervariable region V6 partially and V7, V8, and V9 completely (Table S1 in the Appendixe 1).

Looking to taxonomic results achieved with the RDP classifier for these 20 sequences, it can be seen that sequences either covering the first half part of the gene or covering the second part of the gene allowed identical taxonomic classifications to the last level (genus), except in two cases (clones 5Pd-c-2 and 5Pd-c-12) for which chimerical sequences were detected (Table S1 in the

Appendixe 1). Moreover, the same classification results were obtained when using these same 20 sequences trimmed to equal sizes: on one hand to cover only hypervariable regions V1 to V3 completely and 10% of V4 and on the other hand to cover 10% of hypervariable region V6 and the full length of V7 to V9 (data not shown). Seen this, the 16 rRNA amplicons cloned to characterize the other 2 bacterial consortia (grown without Pd(II) and with 50 mg L^{-1} Pd(II)) were sequenced just with the T7 universal primer.

Among all 42 sequences used in this work to characterize the bacterial communities (Tables S1, S2, and S3 in the Appendix 1), only one did not cover completely either one or the other of these regions. The exception is the sequence of clone 5Pd-c-6, for which a part spanning region V1 and about 9% of region V2 was cropped in the quality inspection step. Nevertheless, this sequence corresponds to an amplicon from one of the groups of sequenced amplicons with identical gel migration patterns and its classification was like the others from that group (order *Clostridiales*, family *Clostridiaceae*), excluding those deciphered to be chimeric.

Characterization of communities - The first thing that stands out when analyzing the composition of bacterial communities on cultures without Pd(II), with 5 mg L^{-1} Pd(II), and with 50 mg L^{-1} Pd(II) (Table 5-2) is that the percentage of bacteria belonging to the family *Clostridiceae* 1 augments as the amount of Pd(II) increases, 3.70 to 21.43% and to 40.91%, respectively, and that bacteria belonging to genus *Clostridium sensu stricto* is causing this drift, showing strong tolerance to this metal.

In addition, also showing this tolerance, other groups of bacteria not even detected in the cultures without or with 5 mg L^{-1} Pd(II) were found in the culture with 50 mg L^{-1} Pd(II). This applies to genera *Hydrogenoanaerobacterium*, *Clostridium XlVa*, *Alkaliphilus*, and *Solitalea* from families *Ruminococcaceae*, *Lachnospiraceae*, *Clostridiaceae 2*, and *Sphingobacteriaceae*, respectively.

In the opposite direction, some groups of bacteria seem to be affected by the presence of Pd(II) in the growth medium: genera *Oscillibacter*, *Clostridiisalibacter*, *Fervidicella*, *Proteiniphilum*, *Alkalitalea*, and Arcticibacter, respectively, from families *Ruminococcaceae*, *Clostridiaceae 3*, *Clostridiaceae 1*, *Porphyromonadaceae*, *Marinilabiliaceae*, and *Sphingobacteriaceae*, were detected in the culture without Pd(II) and not detected in the cultures with 5 and 50 mg L^{-1} Pd(II). The genus *Parabacteroides* from family *Porphyromonadaceae* also seems to be affected but only by higher concentrations of Pd(II): high percentages of bacteria from this taxa were detected in the consortia grown without Pd(II) and with only 5 mg L^{-1} Pd(II) (25.93 and 39.29%, respectively) and they were not detected in the consortium grown with 50 mg L^{-1} Pd(II).

Table 5-2 - Percentages of clones per taxa Order, Family and Genus for each bacterial community. Classifications retrieved by RDP classifier for 16S rRNA gene cloned amplicons representatives of each pattern type on urea-agarose gels after urea/heat-denaturation (Online Resource 4 - Spreadsheets 1, 2 and 3)

Consortium	**Order**	**% of clones**	**Family**	**% of clones**	**Genus**	**% of clones**	**N.er of clones analysed***
Without Pd(II)	Clostridiales	40,74	*Ruminococcaceae*	29,63	*Oscillibacter*	29,63	27
			Clostridiaceae 3	7,41	*Clostridiisalibacter*	7,41	
			Clostridiaceae 1	3,70	*Fervidicella*	3,70	
	Bacteroidales	40,74	*Porphyromonadaceae*	37,04	*Parabacteroides*	25,93	
					Proteiniphilum	11,11	
			Marinilabiliaceae	3,70	*Alkalitalea*	3,70	
	Sphingobacteriales	18,52	*Sphingobacteriaceae*	18,52	*Arcticibacter*	18,52	
With 5 mg L^{-1} Pd(II)	Bacteroidales	39,29	*Porphyromonadaceae*	39,29	*Parabacteroides*	39,29	28
	Campylobacterales	35,71	*Campylobacteraceae*	35,71	*Arcobacter*	35,71	
	Clostridiales	21,43	*Clostridiaceae 1*	21,43	*Clostridium sensu stricto*	21,43	
	Spirochaetales	3,57	*Spirochaetaceae*	3,57	*Sphaerochaeta*	3,57	
With 50 mg L^{-1} Pd(II)	Clostridiales	86,40	*Clostridiaceae 1*	40,91	*Clostridium sensu stricto*	40,91	22
			Ruminococcaceae	22,73	*Hydrogenoanaerobacterium*	22,73	
			Lachnospiraceae	18,18	*Clostridium XlVa*	18,18	
			Clostridiaceae 2	4,55	*Alkaliphilus*	4,55	
	Sphingobacteriales	13,60	*Sphingobacteriaceae*	13,64	*Solitalea*	13,64	

* Clones with sequences deciphered to be chimeric and classified with confidences lower than 90% for the genus level were not considered.

Moreover, interestingly, the genus *Arcobacter* from family *Campylobacteraceae* is a group that stands for being present in a large proportion (35.71%) in the culture with 5 mg L^{-1} Pd (II) and not being detected in cultures without Pd (II) and with 50 mg L^{-1} Pd (II).

4. DISCUSSION

4.1. Screening of cloned amplicons by urea–agarose gel electrophoresis

The validation of a strategy based on running urea–agarose gel electrophoresis to identify similar cloned 16S rRNA genes and reduce redundancy in Sanger sequencing works aiming to characterize bacterial communities depends on two questions: are the genes classified as similar by their gel migration pattern really similar and, the reverse of this, are the genes considered different really different?

The misclassification of 16S rRNA genes as being similar when in reality they are different can have an important impact in terms of characterization of communities because it can lead to not choose to sequence genes of specimens with a significant presence in the bacterial community. In this work, this did not happen. All amplicons that were found to have an equal gel migration pattern had very similar sequences that retrieved matching taxonomic classifications by the RDP classifier and that stayed in the phylogenetic tree in robust clusters separated from amplicons with other gel migration patterns.

The misclassification of a small number of similar 16S rRNA genes as being different does not pose problems because all those considered different are sequenced, allowing posterior correction. In this experiment, only two examples of this misclassification happen: (1) the clones 50Pd-c-20 and 50Pd-c-16, with gel migration patterns 50Pd-gpt-3 and 50Pd-gpt-6, respectively, were both classified in genus *Clostridium sensu stricto* (Table S2 in the Appendix 1) and (2) the clones noPd-c-14 and noPd-c-28, with gel patterns noPd-gpt-4 and noPd-gpt-5, respectively, were classified in genus *Proteiniphilum* (Table S3 in the Appendix 1). Probably, each of these pairs of sequences is from the bacteria of the same genus but of different species; despite the specie's sequences, polymorphisms were detected in the gel; and the taxonomic classification has been made just to the genus level.

In conclusion, in this work, we verified that the simple fingerprinting method of running urea-/heat-denatured cloned 16S rRNA amplicons on urea–agarose gel electrophoresis allows identifying clones with sequences whose similarities lead to the same taxonomic classifications, at least to the genus level. Therefore, it may be considered that this method is efficient to choose similar 16S rRNA amplicons and avoid redundancy in taxonomic studies and thus that it can be used for that purpose instead of the most applied methods up to now: DGGE, TGGE, SSCP, and ARDRA, which have some drawbacks. The method was then applied to select 16S rRNA clones to characterize bacterial communities grown without Pd(II), with 5 mg L^{-1} Pd(II), and with 50 mg L^{-1} Pd(II).

4.2. 16S rRNA-based taxonomy—gene regions

Though sequence analysis of the 16S rRNA gene has been widely used to perform taxonomic studies, its hypervariable regions exhibit different degrees of sequence diversity and no single hypervariable region is able to distinguish among all bacteria (Chakravorty *et al.*, 2007). Sequencing the entire 1500 bp 16S rRNA gene is necessary when describing a new specie or to distinguish between certain particular taxa or strains. However, generating smaller sequences of about 500 bp length is much less expensive and, for example, for most clinical bacterial isolates the initial 500 bp sequence provides adequate differentiation for identification (Clarridge, 2004). Indeed, many studies have been carried out aiming to discover the most suitable partial 16S rRNA gene regions and primers for their amplification by PCR. Some focused on the identification of single bacterial species or differentiate among a limited number of different species or genus (e.g., Becker *et al.*, 2004; Wilson *et al.*, 2007); others focused on the taxonomic characterization of whole prokaryotic community composition (e.g., Liu *et al.*, 2007; Mori *et al.*, 2014).

In this paper, we give a contribution to knowledge on the subject—gene regions suitable for taxonomic studies. For a group of 10 cloned 16S rRNA gene amplicons bidirectionally sequenced, the same taxonomic classifications were retrieved by RDP classifier with both partial sequences obtained for each of them, with sizes between 477 and 934 bp. Moreover, the same taxonomic classifications were obtained with these 20 sequences trimmed to cover on one hand only hypervariable regions V1 to V3 and about 10% of V4 (~600 bp) and on the other hand just about 10% of region V6 and the all extent of V7 to V9 (~490 bp). Therefore, our results point out to robust taxonomic classifications with either one or the other of these two gene regions. This information can be useful when selecting the gene region target to be used in any taxonomic study based on 16S rRNA genes. Moreover, when considering the characterization of bacterial communities by Sanger sequencing of cloned 16S rRNA genes, knowing that unidirectional sequencing with only one primer flanking the cloning site is enough may have important implications by reducing the sequencing costs to half the value of bidirectional sequencing.

The massively parallel high-throughput sequencing technologies enable deep coverage of samples with 16S rRNA short sequencing reads. Thus, lately, great attention has been given to the development of strategies and methods efficient in using such short reads for taxonomic classifications (e.g., Klindworth *et al.*, 2013; Mizrahi-Man *et al.*, 2013). For example, the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu/), an online platform for high-throughput rRNA analysis, provides aligned and annotated rRNA gene sequence data, along with tools to allow researchers to analyze their own rRNA gene sequences (Cole *et al.*, 2014). It includes the tool that was used for taxonomic classifications in this work, the RDP Classifier, which rapidly assigns sequences into taxa with a bootstrap value as an estimate of confidence for each assignment (Wang *et al.*, 2007). The overall accuracy of the RDP Classifier was estimated by Wang and colleagues (2007) to be above around 95% down to the family level and above around 90% to the genus level, either for 400 bp randomly chosen segments or for full length genes (Table 5-3). In this work, the consistent taxonomic classifications obtained with the RDP Classifier for partial sequences from the beginning and from the end of the 16S rRNA gene, with sizes around 600 and 490 bp, respectively, contribute to confirm this robustness.

Table 5-3- Overall classification accuracy (percentages of tests correctly classified) of the RDP Classifier for 16S rRNA gene sequences [adapted from Wang *et al.*, (2007)]

Sequence length	Order	Family	Genus
Full	97,9	95,1	91,4
400 bp	97,7	94,6	88,7

4.3. Characterization of communities and palladium (II) bioremoval

Few studies have been focused on PGM recovery using mixed cultures and to our knowledge just our group, in this work and in another published in 2013 by Martins and colleagues, studied the use of resistant-bacterial communities instead of pure cultures for remediation of palladium (Table 5-4). The applicability of pure cultures is limited since in an industrial process, it is not easy to maintain the sterile conditions necessary to prevent external microbial contamination. The bacterial performance maintenance for a long time is also a problem due to the susceptibility of pure cultures even to small variations in the conditions. Therefore, the use of mixed cultures in biological metals removal or recovery systems is more realistic for future applications.

Table 5-4- Resume of Pd(II) removal studies reported in literature

Genus or species grown or detected	[Pd(II)] tested or measured (mg L^{-1})	Pd(II) removal, (%)	Bioremoval mechanism	Bibliographic references
Clostridium species (in maijority) and genera *Bacteroides* and *Citrobacter (*also present)	18	60	Presence of live cells (Pd-resistant and able to remove)	Martins *et al.*, 2013
Desulfovibrio desulfuricans	213	88	Pd(II) reducer at the expense of H_2	Yong *et al.*, 2002 Baxter-Plant *et al.*, 2003
Desulfovibrio desulfuricans	50	90	Biosorption	Vargas *et al.*, 2004
Shewanella oneidensis MR-1	50	n.a.	Bioreductive Pd(0) precipitation on *S. oneidensis* MR-1 biomass	De Windt *et al.*, 2005
Shewanella oneidensis MR-1	50	94.8	Bioreductive Pd(0) precipitation on *S. oneidensis* MR-1 biomass	De Corte *et al.*, 2012
Citrobacter braakii	50	38.4	Pd associated with the biomass of fermentative bacteria	Hennebel *et al.*, 2011
E. coli	50	n.a.	Pd(II) reduction mechanism of E. coli was attributed to three different [NiFe] hydrogenases	Depanche *et al.*, 2010
Clostridium pasteurianum BC1	2, 20, 50, and 100	99	Reduce Pd(II) ions to form Pd nanoparticles (bio-Pd) that primarily recipitated on the cell wall and in the cytoplasm	Chidambaram *et al.*, 2010

n.a. - means not applied.

SRB are known to be able to remove metals from wastewaters and have been reported to have potential for Pd(II) removal (Yong *et al.*, 2002; Baxter-Plant *et al.*, 2003; Vargas *et al.*, 2004). Thus, the initial community was enriched in a medium that favors the SRB growth (Postgate E). After that, the Pd(II) bioremoval essays were performed in a growth medium which components were based on the composition of Postgate C, a medium also specific for SRB, but with modifications to guarantee Pd(II) solubility. However, perhaps due to these modifications in the growth medium and/or due to the possibility of the sludge used in the enrichment not being a good source of SRB, the bacterial communities developed during the assays did not show significant sulfate-reducing activity. The reasons for this will not be discussed here as the focus of this paper is not the study of SRB but is the characterization of bacterial communities able to remove Pd(II) from aqueous media.

Our results show that WWTP sludge samples can be a good source to collect bacterial communities Pd(II)-resistant and efficient to remove Pd(II) from aqueous solutions, at least for concentrations of this metal up to 50 mg L^{-1}. In the present work, a bacterial consortium resistant and with ability to remove 98% Pd(II) from media with 50 mg L^{-1} of this metal was identified, while only 60% removal from a culture with 18 mg L^{-1} of Pd(II) was achieved in the first (and so far only) work reporting a mixed bacterial community resistant and able to remove this metal. Looking to works with pure cultures, only two cases of experiments with Pd(II) concentrations higher than 50 mg L^{-1} are found: 88% removal from an initial concentration of 213 mg L^{-1} was achieved with *D. desulfuricans* (Yong *et al.*, 2002; Baxter-Plant *et al.*, 2003) and 99% removal was attained with *C. pasteurianum* BC1 from an initial concentration of 100 mg L^{-1} (Chidambaram *et al.*, 2010). However, in the former case, the work was made with resting cell biomass, and in the second case, the palladium reduction was achieved just in 1 min after mixing the metal solution with the bacterial culture, while in our work, the Pd(II) removal occurred during the growth of the bacterial community.

The community grown with 50 mg L^{-1} Pd(II) led to 91% removal of this metal from the culture medium 21 days after inoculation and 98% removal after 28 days. Bacteria from genera *Clostridium sensu stricto*, *Hydrogenoanaerobacterium*, *Clostridium XlVa*, *Solitalea*, and *Alkaliphilus*, respectively, from families *Clostridiaceae* 1, *Ruminococcaceae*, *Lachnospiraceae*, *Sphingobacteriaceae* 2, and *Clostridiaceae* 2, were detected in this consortium. In a work of this type, carried out with mixed communities of bacteria, it is difficult to know which of them were responsible for metal removal. However, it is important to deepen the knowledge about the composition of bacterial communities able to remove metals. Moreover, bacteria tolerant to the metal and with a good growth and activity in his presence are strong candidates for future works with pure cultures, aiming to identify species with strong potential for biotechnological applications, such as metal bioremediation and biorecovery (e.g., production PdS nanoparticles) and for the study of resistance mechanisms.

The most representative bacteria (40.91%) in the consortium grown with 50 mg L^{-1} Pd(II) and whose representation in the community has increased dramatically with increasing Pd(II) into the culture medium belongs to the *Clostridium genus* (Table 5-2), corroborating the idea of its resistance to Pd(II) and emphasizing its potential for palladium biorecovery, as already reported by other authors. Pure cultures of a specie from this genus (*C. pasteurianum* BC1) have already been used to reduce Pd(II) ions, being the palladium precipitated on the cell wall and in the cytoplasm (Chidambaram *et al.*, 2010). Moreover, colleagues of our research group described a mixed bacterial community mostly composed by *Clostridium* species as resistant to and able to remove Pd(II) (Martins *et al.*, 2013) and other colleagues of our group have recently reported mixed communities able to remove copper, zinc, and iron, in which a Gram-positive population mostly assigned to *Clostridium* spp. initiated metal bioremoval based on sulfide generation from components of the medium (mainly sulfite) but not from sulfate (Alexandrino *et al.*, 2014).

In our work, the strong indication of PdS precipitates formation despite just a slight decrease of sulfate and the growing of a bacterial community mostly composed by bacteria belonging to *Clostridium* genus during the Pd(II) removal leaves open the possibility of metal bioremoval by *Clostridium* spp. based on sulfide generation from other then sulfate components.

Another explanation can be the production of sulfide from sulfate by SRB that were in such small percentages in the bacterial communities that were not detected in the taxonomic analysis. This would also agree with the formation of PdS precipitates and with the slight consumption of sulfate observed when looking to values measured at the end of the experiment [around 3 and 16%, respectively for cultures with 5 and 50 mg L^{-1} of Pd(II)]. For both these concentrations of Pd(II) studied, if the small amounts of sulfate consumed were reduced by SRB, stoichiometrically, the quantity of sulfide produced would have been sufficient to precipitate the all the Pd(II) in the form of PdS. In the culture grown with 5 mg L^{-1} Pd(II), from the initial 2728 mg L^{-1} of sulfate, only 71 mg L^{-1} was consumed. In case that it has been reduced by SRB, 24 mg L^{-1} of sulfide was produced. Stoichiometrically, this makes 15.4 times more sulfide than the necessary to convert all the Pd(II) initially present in this assay (5 mg L^{-1}) in PdS precipitates. For the culture with 50 mg L^{-1} Pd(II), also assuming SRB activity, the 255 mg L^{-1} of sulfate consumed (of the initial 1599 mg L^{-1}) was reduced to 85 mg L^{-1} of sulfide. In this case, the production of sulfide is 5.6 times higher than the necessary to precipitate the Pd(II) initially added to this assay (50 mg L^{-1}).

Together, the much more pronounced decrease of the Eh in biotic tests than in abiotic tests, which suggests that there was biological production of at least one reducing agent, and the strong indication that the precipitates formed in the biotic assays are PdS particles suggest that the reducing agent H_2S was produced by growing bacteria. This corroborates both pointed hypotheses of being Clostridium spp. or SRB the responsible agents for the biological removal of Pd(II) from the culture medium.

As for the other bacterial genera detected in the consortium grown with 50 mg L^{-1} Pd(II), they are known to be adapted to extreme environments, but it is the first time they are referred as palladium resistant. *Clostridium XlVa* has been reported among the most abundant genus occurring in studies regarding remediation of acid mine drainage (AMD) (Sánchez-Andrea *et al.*, 2014), which is characterized by being contaminated with high concentrations of metals. Concerning *Hydrogenoanaerobacterium* as metal-resistant bacteria, no references were found in the literature. Nevertheless, this taxonomic group has been identified as butanol- and isobutanol-tolerant bacteria (Kanno *et al.*, 2013). *Solitalea* bacteria present several properties, including anaerobic growth and nitrate reduction. These bacteria have been detected in extreme environments such as in a denitrification reactor and in saline-alkaline lakes (Zhu *et al.*, 2015; Silva, 2015). Moreover, solitalea-like bacteria have been described as not being influenced by antibiotic (neomycin and streptomycin) treatments (Kopecky *et al.*, 2014), which is another evidence of their resistance to extreme conditions. The *Alkaliphilus* species usually survive in certain extreme environments. For example, the *Alkaliphilus transvaalensis* are strictly anaerobic and extremely *alkaliphilic* (Kobayashi *et al.*, 2007) and some *Alkaliphilus* species are alkaliphilic metal-reducing bacteria [Fe(III), Cr(VI), Co(III), U(VI) and Se(VI)], as *Alkaliphilus metalliredigens* (QYMF), and have been used in metal reduction and biomineralization processes (Roh *et al.*, 2007). Thus, these authors' results together with our results suggest that all these groups of bacteria are resistant to various types of extreme environments, which make them interesting as targets for the study of resistance mechanisms and eventually for biotechnological applications, such as metals bioremediation and biorecovery.

In the bacterial community grown with 5 mg L^{-1} of Pd (II), beyond bacteria from the genus Clostridium, the Parabacteroides genus was the predominant, followed by the *Arcobacter* genus. These two bacteria genus were not identified before as resistant to Pd(II); thus, they are also candidates to be further studied as putative efficient Pd(II) removal agents.

Moreover, the detection of a high percentage (35.71%) of bacteria from genus *Arcobacter* only in the consortium grown with 5 mg L^{-1} of Pd(II) is very interesting because this suggests that for this taxa, the presence of some Pd(II) in the growth medium is more favorable than not having any of this metal at all. The research carried out by Fedorovich and colleagues (2009a, 2009b) in the development and optimization of a microbial fuel cell (MFC) revealed another clue pointing to a favorable use of palladium by some *Arcobacter*. MFC are anaerobic biological systems developed to generate electricity in which microorganisms transfer electrons from organic compounds to a conductive external electron acceptor provided by an artificial anode connected to an electric circuit. From our point of view and given our results, it is interesting that an *Arcobacter* strain (*A. butzleri* strain ED-1) has been selectively enriched specifically associated with theMFC electrode (Fedorovich *et al.*, 2009a) and that the electrode comprise porous graphite plates coated with palladium (Fedorovich *et al.*, 2009b). So, the hypothesis that in anaerobic conditions, the presence of Pd(II) in the medium favors the proliferation of some *Arcobacter* can be proposed. Accepting this hypothesis as true, the absence of *Arcobacter* in the consortium grown with 50 mg L^{-1} Pd(II) can be due to the toxicity of the metal ion at that concentration.

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Compliance with ethical standards

Conflict of interest - All authors declare that they have no competing interests.

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CHAPTER 6

A bridge between liquid-liquid extraction and the use of bacterial communities for palladium and platinum recovery as nanosized metal sulphides

ABSTRACT

The Platinum Group Metals (PGM) are rare in the Earth's crust and in the past years their use had a considerable expansion limiting their availability, which justifies PGM's high commercial value and demand. Therefore, PGM recovery from secondary sources is very important from both economic and environmental points of view. In recent years, several methods for PGM removal have been investigated. Our research group has been studying the removal/recovery of PGM using both chemical (liquidliquid extraction) and biological methods (using anaerobic bacterial communities). The aim of this study was to combine these two chemical and biological approaches technologies, for PGM recovery. For that purpose, Pd(II) and Pt(IV) present in aqueous phases, were extracted to organic phases composed by N,N'-dimethyl-N,N'-dicyclohexylsuccinamide (DMDCHSA) and N,N'-dimethyl-N,N'-dicyclohexyltetradecylmalonamide (DMDCHTDMA) in 1,2-dichloroethane, respectively, with an extraction efficiency of 79% for Pd(II) and 99% for Pt(IV). The metals in the loaded organic phases were then stripped with seawater, a low-cost solution largely available in nature, with efficiencies of 100% for Pd and 86% for Pt. Finally, the metals were precipitated and recovered using metabolic products produced by a community enriched for sulphate-reducing bacteria. The palladium precipitated completely, while 86% of platinum was precipitated. During the precipitation process sulphide concentration in the solution decreased and the analysis of the precipitates is consistent with the formation of nanosized PdS and PtS_2.

To our knowledge, this research shows, for the first time, the potential of combining liquid-liquid extraction with the use of bacteria aiming platinum and palladium recovery, as metal sulphides, from aqueous media.

Keywords: Liquid-liquid Extraction; Bacterial community; Metabolic products; Palladium; Platinum; Nanosized palladium and platinum suphides

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1. INTRODUCTION

Platinum Group Metals (PGM), including platinum and palladium, are a limited global resource, not only because they are rare in the Earth's crust, but also due to the increasing of their use as a primary raw material, resulting in the high PGM economic value and demand (Martins *et al.*, 2013; Deplanche *et al.*, 2014). The exceptional properties of PGM, such as high conductivity, thermal stability and catalytic properties (Loferski, 2011), turn the PGM attractive to many industries, particularly in electronic devices industries, in chemical processes industries, as catalysts, and in automotive industry, as catalytic converters. In this context, PGM recycling from manufactured materials is essential and can provide a secondary source of these metals (Butler, 2012). Over the last years, the recovery and reutilization of PGM from industrial wastes has raised some interest and several methods have been pointed out and explored, such as chemical reduction (Konishi *et al.*, 2007), electrochemical methods (Dean, 1979) and biological methods (Martins *et al.*, 2013). However, not all methods achieve an efficient separation and recovery of PGM. For instance, the fraction of PGM recovered from spent catalysts by liquid-liquid extraction, achieves only around 40% of the total amount (Marinho *et al.*, 2010). Among the chemical methods, liquid-liquid extraction, also called solvent extraction (SX), has been used in nuclear chemistry technology for the separation of various radioisotopes and for the reprocessing of nuclear fuels (Lee *et al.*, 2009a), or for integrating hydrometallurgical schemes for the recovery of metals from mineral ores or from secondary sources (Marinho *et al.*, 2010). Moreover, SX has been studied for PGM recovery due to its advantages, as high selectivity and metal purity. The SX processes, allowing the separation of two or more components in two immiscible liquid phases, are usually used by hydrometallurgical industries for PGM recovery, because their metal ions can easily form species which are extractable into the organic phase (Butler, 2012; Rao *et al.*, 2000) with various reagents. Di-n-octyl sulphide or hydroxyoxime are examples of selective extractants for palladium while tributyl phosphate (TBP) and amines are frequently mentioned for platinum (Rydberg *et al.*, 2004) among others (Harjanto *et al.*, 2006; Barakat *et al.*, 2006; Komjarova and Blust, 2006; Costa *et al.*, 2013). Recently, Costa and colleagues (2013) proposed a new promising extractant (N,N´dimethyl-N,N´-dicyclohexyltetradecylmalonamide, DMDCHTDMA, for the recovery of platinum (IV) from hydrochloric acid media. The platinum can be easily and quantitatively stripped to a new aqueous phase by simple contact with a 1 M HCl solution. Furthermore, this extractant showed a good loading capacity towards platinum (IV) and it can be reutilized several times without losing its extraction ability (Costa *et al.*, 2013). In addition, the same research group has very recently discovered the extraction ability of N,N'-dimethyl-N,N'dicyclohexylsuccinamide, DMDCHSA, for the extraction on both Pt(IV) and Pd(II) from hydrochloric acid media (data not published). Taking into account the good performance of these two extractants they were both selected and used in the present investigation. The PGM stripping from the loaded organic phase and the subsequent recovery is not so much studied. The metals recovery from the loaded organic phase by stripping methods is usually a difficult process, especially when the metals concentration is low, which is normally the case of PGM. The use of Na2S2O3 (Nowottny *et al.*, 1997) or thiourea (Lee *et al.*, 2009b; Kumar *et al.*, 2008; Lee at al., 2009a) as stripping agents for platinum in a single stage has been explored (Marinho *et al.*, 2010), but the replacement of these chemicals by more environmentally friendly and low-cost alternative stripping agents still remains a relevant issue. The use of biological methods mainly based on the use of bacterial strains and communities for PGM recovery from aqueous solutions has also been investigated (Martins *et al.*, 2013). The PGM biorecovery processes have some advantages compared to the chemical methods, since the biorecovery strategies are cleaner and more attractive from an economic point of view and particularly suitable for metals recovery at low concentrations (Macaskie *et al.*, 2010). Several studies have shown the potential of pure bacterial cultures for biological reduction of Pd(II) to Pd(0), such as Desulfovibrio desulfuricans (Yong *et al.*, 2002) and Shewanella oneidensis (De Windt *et al.*, 2005) and platinum bio-reduction by Shewanella algae (Konishi *et al.*, 2007), cyanobacteria (Lengke *et al.*, 2006; Brayer *et al.*, 2007) and Desulfovibrio sp. (Rashamuse and Whiteley, 2007; Rashamuse *et al.*, 2008). Although the use of bacterial communities presents considerable recognised advantages over the use of pure strains (Mukred *et al.*, 2008), few studies are reported. A Sulphate-Reducing Bacteria (SRB) community was mentioned as having the ability to reduce Pt(IV) to Pt(0) via the intermediate cation Pt(II) (Riddin *et al.*, 2009). Other authors demonstrated the ability of a Pd(II)-resistant mixed bacterial culture to recover palladium (II) from an aqueous medium, leading to the formation of Pd(0) nanoparticles (Martins *et al.*, 2013). Palladium and platinum bioremoval mechanisms, such as the bioaccumulation of palladium by Desulfovibrio fructosivorans (Mikheenko *et al.*, 2008) and the bioabsorbance of Pd and Pt by Desulfovibrio (Vargas *et al.*, 2004) have been reported. In the present research we intend to test the potential of metabolic products (MP) from the growth of an anaerobic bacterial community, enriched for SRB, for Pd and Pt removal from aqueous solutions, namely those resulting from the stripping stage of SX processes. The use of MP presents advantages in comparison to the bioremoval techniques that occur in the presence of live bacterial cells, since the process does not require the utilization of metals-resistant bacteria. The combination of both chemical (SX) and biological methods may have potential for PGM recovery, taking into account that after the stripping stage of a solvent extraction process, the metals remain soluble in the purified aqueous phase, and thus a subsequent method is required for its recovery. Hence, this research explores for the first time, to our knowledge, the possibility of combining the use of liquid-liquid extraction with a biological process for the recovery of platinum and palladium from aqueous media.

2. MATERIAL AND METHODS

2.1. Synthesis and characterization of the extractants

The structures of the malonamide and succinamide derivatives synthesized and used in this research work, N,N'-dimethyl-N,N'-dicyclohexyltetradecylmalonamide (DMDCHTDMA) and N,N-dicyclohexyl-N,N-dimethylsuccinamide (DMDCHSA), are presented in Figure 6-1.

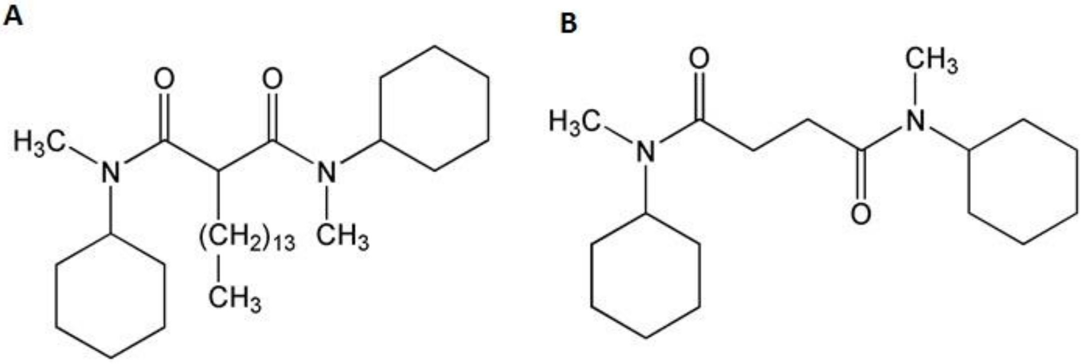

Figure 6-1 - Molecular structure of the DMDCHTDMA (A) and DMDCHSA (B).

DMDCHTDMA was synthesized according to the procedure already described by Costa and colleagues (2013) and its characterization was in accordance with the data previously reported. The reagents used for the DMDCHSA synthesis were succinyl chloride (Sigma-Aldrich, 95%), N-methylcyclohexylamine (Sigma-Aldrich, 99%), and dichloromethane (DCM, Absolve, ≥ 99%) and were used without further purification. Succinyl chloride (7g, 5mL, 0.044 mol) was dissolved in 75 mL of DCM in a two neck round bottom flask, and cooled down in a cold bath (ice/$CaCl_2$ -15°C). N-methylcyclohexylamine (20.83g, 24 mL, 0.184 mol) was dissolved in 25 mL of DCM, in an addition funnel. The amine solution addition was performed dropwise under stirring. The reaction was monitored by Fourier Transform Infrared Spectroscopy (FTIR) in a Bruker (Tensor 27) spectrophotometer, placing an aliquot into NaCl disks. The disappearance of the band at 1783 cm^{-1} (acyl chloride carbonyl group) and the appearance of the amide carbonyl band at 1639 cm^{-1} were monitored. After 48 h, the reaction was completed and the obtained mixture was transferred to a separatory funnel, washed three times with HCl solution (4% w/w)), once with distilled water and, finally, dried over anhydrous magnesium sulphate and filtered to a round bottom flask. The DCM was evaporated in a rotary evaporator, yielding a yellow solid that was recrystallized from *n*-hexane and dried in a vacuum oven at 30°C overnight. The yield of the product (DMDCHSA) was 80%, corresponding to 11.14 g of compound. DMDCHSA was characterized by melting point (86-88°C), FTIR (KBr: 1639 cm^{-1} (ν C=O), 1591 cm^{-1} (Amide II)), ^{1}H NMR($CDCl_3$: 4.44 (1 H, broad singlet, CH cyclohexyl), 3.69 (1 H, broad singlet, CH cyclohexyl), 2.88 (3 H, s, N-CH_3), 2.81 (3 H, s, N-CH_3), 2.73-2.65 (4 H, m, O=C-CH_2-CH_2-C=O), 1.85-1.05 (20 H, m, CH_2 cyclohexyl), and LC-MS (*m/z*: 309 ($[M+H]^+$)), the spectroscopic data being consistent with the expected structures.

2.2. Solvent extraction and stripping experiments

All reagents and solvents were used without further purification. Feed aqueous phases containing 102 mg L^{-1} Pd(II) or 163 mg L^{-1} Pt(IV) in HCl 6M (prepared from a hydrochloric acid, 37% (w/w) solution, ACS reagent, Acros Organics) were prepared from their respective atomic absorption spectroscopy standards (1 g L^{-1} Pd(II), Palladium standard solution, CertiPUR, Merck or 1g L^{-1} Pt(IV), Platinum Standard solution, Acros Organics) and were dissolved in the required volumes of the hydrochloric acid solution. The organic phases containing 0.05M DMDCHSA and 0.05 M DMDCHTDMA were prepared in 1,2-dichloroethane (1,2-DCE, VWR Prolabo BDH chemicals). The aqueous phases containing Pd(II) or Pt(IV) were then placed in contact with the organic phases. Seawater and 1M hydrochloric acid solution were used as agents to strip palladium and platinum from the respective loaded organic phases. The extraction and stripping experiments were carried out at room temperature and the organic and aqueous phases were placed in contact with equal volumes (A/O = 1) during 30 minutes, by mechanical shaking, adopting a rotation speed between 900 and 1000 rpm. The two phases were separated and the aqueous phases were filtrated with a filter paper (silicone treat filter paper, Whatman, 1PS). The palladium and the platinum concentrations in the aqueous solutions were determined by flame atomic absorption spectroscopy (Flame-AAS) using an Analytic Jena novAA 350 model spectrometer. Four replicates were carried out for each metal (Pd(II) and Pt(IV)) in the extraction experiments. In the stripping experiments two replicates were performed for each stripping agent and for each metal. The maximum coefficient of variance estimated for the results achieved from the different extractions and stripping experiments was approximately ± 5%.

2.3. Pd and Pt biorecover from the metal loaded stripping phases with metabolic products

The metabolic products (MP) were obtained from an anaerobic bacterial community mostly composed by bacteria close to the genus Oscillibacter (32%), Parabacteroides (28%) and Arcticibacter (20%), according to previous 16S rRNA gene taxonomic analysis. The community was previously enriched for SRB from a sludge sample from the Wastewater Treatment Plant (WWTP) of Lagos in southern Portugal. The bacterial enrichment was performed at room temperature (21 ± 1°C) in 120 mL glass bottles in anaerobic conditions achieved by purging the medium with nitrogen gas and addition of 10 mL of sterile liquid paraffin (Martins *et al.*, 2010). The medium used for this enrichment was Postgate E medium (Postgate, 1984), typically used for SRB, with some modifications: 0.5 g L^{-1} KH_2PO_4, 1 g L^{-1} NH_4Cl, 2.5 g L^{-1} Na_2SO_4, 0.06 g L^{-1} $CaCl_2.6H_2O$, 1 g L^{-1} yeast extract, 0.0072 g L^{-1} $FeSO_4.7H_2O$, 0.06 g L^{-1} $MgSO_4{\cdot}7H_2O$, 0.3 g L^{-1} trisodium citrate dehydrate, 0.1 g L^{-1} ascorbic acid, 0.081 mL thioglycolic acid and supplemented with resazurin as redox indicator (0.03 g L^{-1}) and sodium lactate (6.0 g L^{-1}). For the enrichment, a sludge volume of 10 mL in 90mL of culture medium was used. The pH was maintained at 7.2 ± 0.3. The culture was allowed to grow for 4 weeks and was subsequently inoculated in the same conditions and in the same medium without resazurin and finally the pH of the medium was adjusted to 6.7 ± 0.2 with diluted solutions of NaOH or HCl. In each inoculation assay, 10% (v/v) of inoculum from the enrichment assays was used. For that purpose, the bacterial cells were harvested from the enrichment by centrifugation at 4000 rpm for 10 min and washed with growth medium. The culture was carried out in anaerobic conditions. The medium was purged with nitrogen gas, in order to reach the anaerobic conditions and after inoculation 10 mL of liquid paraffin was added. The bottles were sealed with butyl rubber stoppers and aluminium seals and the experiments were incubated at room temperature. The bacterial culture was allowed to grow to achieve an OD_{600} around 0.6-0.8 and then a 30 mL sample of each batch was collected and centrifuged at 4000 rpm for 10 min and filtered through a 0.22 µm filter (syringe filter, Puradisc FP 30, Whatman). 10 mL of the filtrate, containing Metabolic Products (MP) produced during bacterial growth, was placed in 30 mL glass bottles and then the same volume (10 mL) of the rich Pt and Pd aqueous phases resulting from the stripping step were added. The bottles were sealed with butyl rubber stoppers and aluminium seals. The experiments were performed in duplicate at room temperature. Samples were collected immediately after the addition of the Pt or Pd stripping loaded phase to the solution containing the MP and weekly during 21 days to analyse pH, redox potential (Eh), sulphate, sulphide and palladium or platinum concentrations.

Summarizing, the whole process of extraction and biorecovery of Pd and Pt is composed by three steps, two of each representing the liquid-liquid extraction stage (Extraction and Stripping steps) and the last one the Biological step (Pd and Pt biorecovery using MP). The following diagram representing the whole process is shown in Figure 6-2.

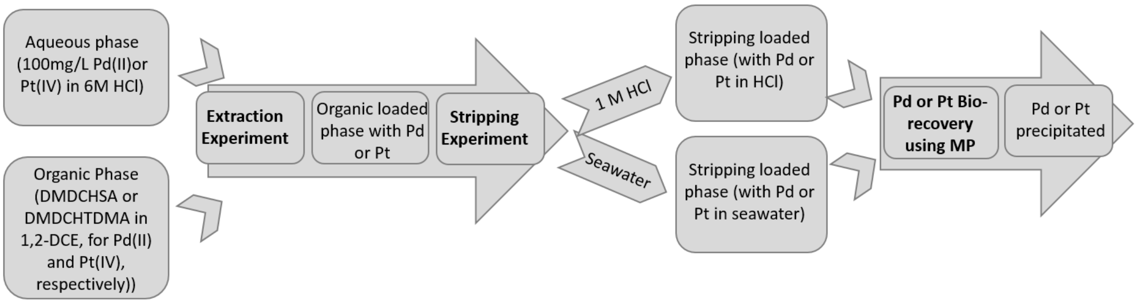

Figure 6-2 - Process diagram. The three steps of the process: Extraction, Stripping and Pd or Pt biorecovery using MP, are represented in bold.

2.4. Analytical methods

Samples from batch cultures were periodically collected using a sterile syringe and in each sample, immediately after sampling, sulphide concentration was determined by the Methylene Blue Method (665 nm, Hach-Lange) and the optical density at 600 nm (OD_{600}) was measured, in both cases using a UV–Visible spectrophotometer (DR 2800, Hach-Lange. Then, after centrifugation at 4000 rpm for 5 min, the samples were analysed. Redox potential and pH were determined using a pH/E Meter (GLP 21, Crison) and sulphate concentration was quantified by UV-visible spectrophotometry (Hach-Lange DR2800 spectrometer) using the method of SulfaVer®4.

2.5. Precipitates characterization

The precipitates were obtained by centrifugation (4000 rpm, 10 min) and the pellets were washed 3 times with ethanol 70% (stirred and centrifuged 4000 rpm 20min) and then the precipitates were dried under vacuum (Binder, VDL) at 37°C ± 1°C. The precipitates were analysed by X-Ray Diffraction (XRD) using a PANalytical X'Pert Pro powder diffractometer and by Scanning Electron Microscopy (SEM, Hitachi, 3700N) (concept of the techinique in Appendix 3) coupled to an Energy Dispersive X-ray Spectrometer (EDS, Bruker, Xflash 5010), (SEM-EDS) (concept of the technique in Appendix 3). In order to confirm the information obtained by SEM-EDS and the particles size, a Transmission Electron Microscopy (TEM, Hitachi, model H8100, with LaB6 filaments) analysis was made. This analysis was also coupled to an Energy Dispersive X-ray Spectrometer (EDS, ThermoNoran) aiming to determine the elemental composition of the precipitates.

3. RESULTS AND DISCUSSION

Our research group has dedicated part of its work to the investigation of the removal/recovery of PGM using both chemical and biological methods. The chemical methods for PGM removal developed in our laboratory have been focused in the use of amides for liquid-liquid extraction, while the biological methods have been based on the usage of anaerobic bacterial communities. Therefore, it was decided to test the combination of these two technologies for PGM removal in order to take advantage of both.

3.1. Liquid-liquid extraction of platinum (IV) and palladium (II) from hydrochloric acid solution

Platinum (IV) and palladium (II) extractions were carried out according to previous tests performed by our group, as mentioned above, in which the chosen extractants, DMDCHTDMA and DMDCHSA, demonstrated to be good platinum (Costa *et al.*, 2013) and palladium (unpublished results) extractants, respectively and. The results obtained in the experiments using 0.05 M DMDCHTDMA in 1,2-DCE as organic phase and 6 M hydrochloric acid with platinum (IV) as aqueous phase showed an efficiency of platinum (IV) extraction (%E) of 99.3 ± 0.3, corresponding to a final Pt(IV) concentration in the organic phase of 161 mg L^{-1}. These results are similar to those obtained by Costa and colleagues (2013), that considered DMDCHTDMA a promising extractant for the recovery of Pt(IV) from hydrochloric acid media and able to extract around 88% Pt(IV) in the same conditions. The results obtained in the SX assays performed with 0.05 M DMDCHSA in 1,2-DCE as organic phase and 6 M hydrochloric acid with palladium (II) as aqueous phase showed an efficiency of palladium (II) extraction, (%E), of 78.6 ± 1.4, corresponding to a final Pd(II) concentration in the organic phase of 81 mg L^{-1}.

3.2. Stripping of platinum and palladium from the loaded organic phases

In a SX process it is essential to find an effective aqueous solution to strip the metals present in the loaded organic phase. For that purpose, 1 M HCl, a known efficient stripping agent for platinum (IV) (Costa *et al.*, 2013), was used in this case for both platinum (IV) and palladium (II). According to previous studies (Costa *et al.*, 2013; Costa *et al.*, 2004) the presence of chloride ion seems to be essential for the effectiveness of the stripping step due to the formation of Pt and Pd stable chloro-complexes, which allows the transference of those metals from the organic to the new aqueous phase. Taking that into account and considering the need of using environmentally friendly and low-cost stripping agents also widely available in nature, the use of seawater was regarded as very convenient. Thus, seawater, very rich in chloride ions, typically with a Cl^{-} concentration around 0.55 M, was also tested as stripping agent and its efficiency was compared with the stripping efficiency of the 1M HCl solution. Each of the two loaded organic phases containing 161 mg L^{-1} Pt(IV) and 81 mg L^{-1} Pd(II) were contacted with each stripping agent (1 M HCl or seawater) for 30 minutes with stirring and A/O = 1. No third phase was detected and the phase's separation was fast. The stripping percentages, (%S), obtained for Pt(IV) with 1 M HCl solution and with seawater were 59.7 ± 4.8 (97 mg L^{-1}) and 85.8 ± 0.8 (138 mg L^{-1}), respectively. The result obtained using 1 M HCl as Pt stripping agent, around 60%, was lower than that previously reported by Costa and colleagues (2013). However, it can be concluded that Pt(IV) can be easily and effectively stripped from the loaded DMDCHTDMA organic phases simply using seawater, an inexpensive and environmental friendly solution. Another advantage of DMDCHTDMA aiming at its potential industrial application is the possibility of this extractant to be reused after several extraction and stripping cycles, as reported by Costa and colleagues (2013). This behaviour was also observed for DMDCHSA (data not published). Regarding the results obtained for Pd(II) with both stripping agents (1 M HCl and seawater), the %S was 100%, which corresponds to 81 mg L^{-1} of Pd(II) in each loaded stripping phase. These results demonstrated that it is irrelevant to use 1 M HCl or seawater as stripping agent, although the utilization of seawater offers important economic and environmental advantages. According to our knowledge, seawater is being mentioned for the first time as a potential stripping agent to be eventually used in industry for the re-extraction (stripping) of palladium or platinum from a loaded organic phase resulting from SX.

3.3. Bacterial enrichment and re-inoculation

The metabolic products (MP) were obtained from an anaerobic bacterial community previously enriched for SRB from a sludge sample from a WWTP and then re-inoculated. The bacterial growth during re-inoculation was monitored for 4 weeks and the optical density at 600 nm (OD_{600}) and the redox potential (Eh) results are shown in Figure 6-3.

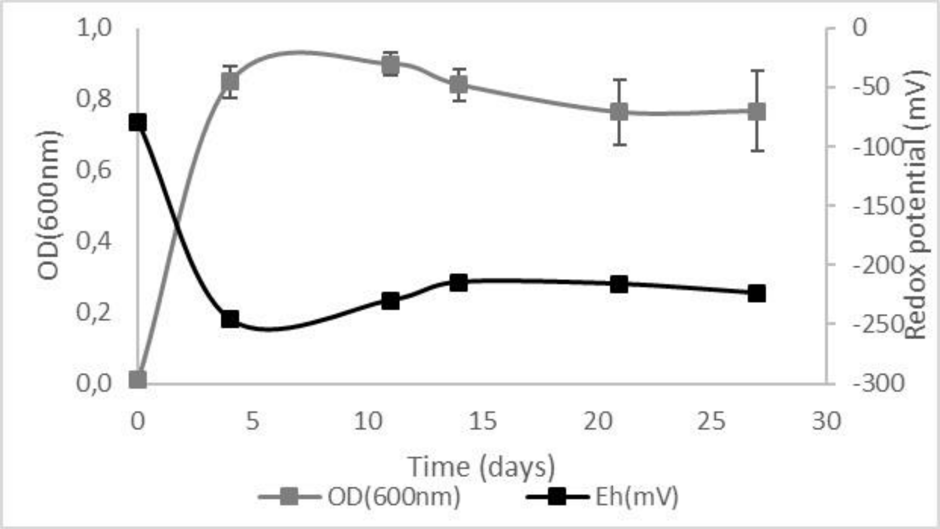

Figure 6-3 - Growth parameters (optical density at 600nm – grey square - and redox potential – black square) of the bacterial community used for collecting the metabolic products. Data are the average of duplicates and error bars indicate the standard deviations of the average values (in some cases error bars are smaller than symbols, therefore not visible).

According to the results presented in Figure 6-3, OD_{600} increases while Eh decreases and the pH remained constant (6.7 ± 0.2) (data not shown) contributing to the excellent growth. The decrease of the redox potential is probably related to oxygen consumption, to sulphate reduction to sulphide and to other metabolic reactions occurring during anaerobic bacterial growth. The log phase achieved the maximum (OD_{600}= 0.8-0.9) in a short time (4-5 days) with the simultaneous Eh decrease, indicating the well growth of the bacterial community. In the stationary phase, after the 5th day, the redox potential remained more or less constant to the end of the assay. Although the sludge enrichment has been designed to promote the growth of SRB, these bacteria were not identified by 16S rRNA gene taxonomic analysis, due to the fact that SRB were present in minority. In fact, the presence of SRB was confirmed by dsr gene (data not shown), that has proven to be a good alternative in the case of SRB taxonomic identification (e.g. Wagner et al, 1998; Karr et al, 2005). This is consistent with the low sulphate reduction, from 2300 mg L^{-1} to 1600 mg L^{-1} during the bacterial growth, leading to approximately 230 mg L^{-1} of sulphide production.

3.4. Pd and Pt recovery using metabolic products

After 4 weeks growth, the MP were collected, filtered and placed in glass bottles and then the stripping loaded phase with Pd or with Pt, resulting from the stripping assays using seawater (mentioned above), was added. The volume ratio of both solutions was 1:1, being the solutions diluted to half. The MP were analysed before addition of the stripping loaded phase and presented a pH of 7.6 and a Eh of -332 mV. The sulphate and sulphide concentrations were 1590 mg L^{-1} and 225 mg L^{-1}, respectively. The mixtures composed by the MP and the metal loaded solution resulting from stripping, with 795 mg L^{-1} sulphate, 112.5 mg L^{-1} sulphide and 40.5 mg L^{-1} of Pd or 69 mg L^{-1} of Pt, were maintained in batch for 21 days. During this time, pH, Eh, sulphide and platinum or palladium concentrations were monitored. The pH remained constant, 7.1 ± 0.3 in the experiment with palladium and 7.4 ± 0.2 in the experiment with platinum and the Eh increased in both assays. The results obtained for Pt and Pd removal from the stripping phase and respective sulphide concentrations are presented in the Table 6-1.

Table 6-1 - Sulphide, palladium and platinum concentrations along bioremoval experiment (Pd and Pt stripping loaded phase and metabolic products) and final Pd and Pt removal percentage

	MP (before addition)		**Stripping loaded phase (before addition)**		**Mixture (stripping loaded phase + MP) (1:1 (v/v))**		**Mixture (after adding the stripping loaded phase to the MP)**			
Concentration	**mg/L**	**mM**	**mg/L**	**mM**	**mg/L**	**mM**	**t= 0.0625 days**	**t= 3 days**	**t= 7 days**	**Final metal removal (after 21 days) (%)**
			HCl (stripping agent)				**mg/L**	**mg/L**	**mg/L**	
[Sulphide]	225	7.0	-	-	112.5	3.5	32.7 ± 4.9	16.1 ± 1.0	10.1 ± 1.5	
[Pd]	-	-	81	0.8	40.5	0.4	1.8 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	100
[Sulphide]	225	7.0	-	-	112.5	3.5	44.4 ± 5.8	26.3 ± 1.9	22.7 ± 2.1	
[Pt]	-	-	97	0.5	47	0.2	3.1 ± 0.0	0.4 ± 0.1	0.5 ± 0.1	99
			Seawater (stripping agent)							
[Sulphide]	225	7.0	-	-	112.5	3.5	18.8 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	
[Pd]	-	-	81	0.8	40.5	0.4	14.5 ± 3.0	0.5 ± 0.2	0.0 ± 0.0	100
[Sulphide]	225	7.0	-	-	112.5	3.5	36.6 ± 0.1	0.5 ± 0.2	0.0 ± 0.0	
[Pt]	-	-	138	0.7	60.5	0.3	21.7 ± 5.6	20.4 ± 1.9	12.2 ± 0.1	88

The results shown in Table 6-1 suggest that most of the platinum and palladium removal occurs immediately after the addition of the metal loaded stripping phase to the MP solution. The best metal removal was achieved for palladium, independently of the loaded stripping phase (1M HCl or seawater) used, reaching up to 100% removal of Pd in less than 4 days. Pt removal results are also very promising since removal percentages of 99% and 88% were obtained with 1M HCl and seawater, respectively. Nevertheless, considering that seawater, comparing to 1 M HCl, presents economic and environmental advantages, the percentage of platinum removal obtained (88%) is a very promising result. Palladium and platinum removal is accompanied by consumption of sulphide from the solution, which may indicate that both metals were removed as insoluble metal sulphides. Despite the low reduction of sulphate to sulphide achieved by the bacterial community (30%), this reduction is enough for palladium and platinum removal from the medium. As shown in Table 6-1, the sulphide concentration in the mixture of MP + stripping loaded phase was 3.5 mM, while the concentrations of Pd and Pt were 0.38 and 0.31 mM, respectively. Thus, the concentrations of sulphide required in the mixture for the complete precipitation of PdS and PtS_2 is 0.38 mM and 0.62 mM, lower than those present in the solution. Table 6-2 presents a summary of the results obtained for Pd and Pt in the three steps (extraction, stripping and biorecovery) that compose the whole process previously described in Figure 6-2.

Table 6-2 - Extraction and bioremoval results for palladium and platinum along the three subsequent experiments (Metals Extraction to an organic phase, Metals Stripping to 1 M HCl or seawater and finally Metals bioremoval using MP)

Assays		**Extraction**			**Stripping**			**Metals Biorecovery using MP (1:2 dilution)**	
Metal	**Metals initial concent. (mg/L)**	**Extractant**	**% Extraction**	**Final metals concent. (mg/L)**	**Stripping agent**	**% Stripping**	**Final metals concent. (mg/L)**	**% Removal**	**Final metals concent. (mg/L)**
Pd	102	DMDCHSA	79	81	1M HCl	100	81	100	40.5
					Seawater	100	81	**100**	**40.5**
Pt	163	DMDCHTDMA	99	161	1M HCl	60	97	99	48.0
					Seawater	86	138	**88**	**60.5**

In order to assess the chemical composition of the precipitates, their characterization by X-Ray Diffraction (XRD) was performed. The analysed palladium and platinum precipitates were obtained after contact of the stripping loaded phases with the metabolic products. The stripping loaded phases chosen were those in which seawater was used as stripping agent because of the greater advantages, already mentioned associated with their use. The XRD results for both Pt and Pd precipitates were inconclusive (data not shown), due to a large number of peaks in the diffractogram. Those peaks were probably due to the presence of salts from the culture medium and/or from seawater. However, SEM-EDS and TEM-EDS analysis were also carried out and revealed important information about the samples, including external morphology, size and elemental composition, offsetting the absence of XRD information. The analysis of the precipitate obtained in the assay with the platinum loaded stripping phase (seawater) by SEM and XEDS mapping is showed in Figure 6-4.

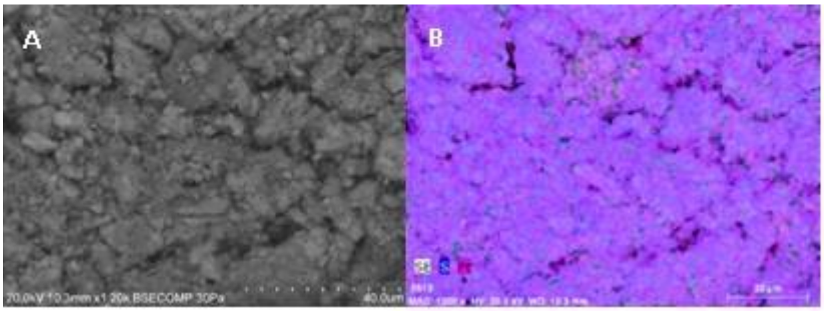

Figure 6-4 - SEM image (A) and XEDS mapping (B) of the precipitates obtained in the batch assays in which platinum loaded stripping phase (seawater) was added to the metabolic products.

The SEM results, Figure 6-4A, showed that the precipitates are agglomerated; however it was not possible to conclude about the particles morphology. The mapping micrograph, Figure 6-4B, showed an overlap of Pt and S elements, which is consistent with the formation of platinum sulphide. The SEM-EDS analysis (Figure S8 in the Appendix 2) identified elements present in the culture medium and in seawater such as Ca, Mg, and Na, although the more intense peaks belong to Pt and S. The atomic percentages of Pt and S in the precipitate is 9.7% and 58.6%, respectively, which corresponds to an approximate stoichiometry of 1Pt to 6S, higher than that expected for PtS_2. This ratio can be explained by the excess of sulphide in the MP in relation to platinum in the mixed solution, 0.62 mmol Pt to 3.5 mmol sulphide, when just 1.24 mmol of S is needed for complete precipitation of PtS_2. TEM analysis (Figure 6-5) allowed achieving a higher magnification, thus confirming that the particles are in clusters (agglomerated) and have variable shape and size (30-150nm).

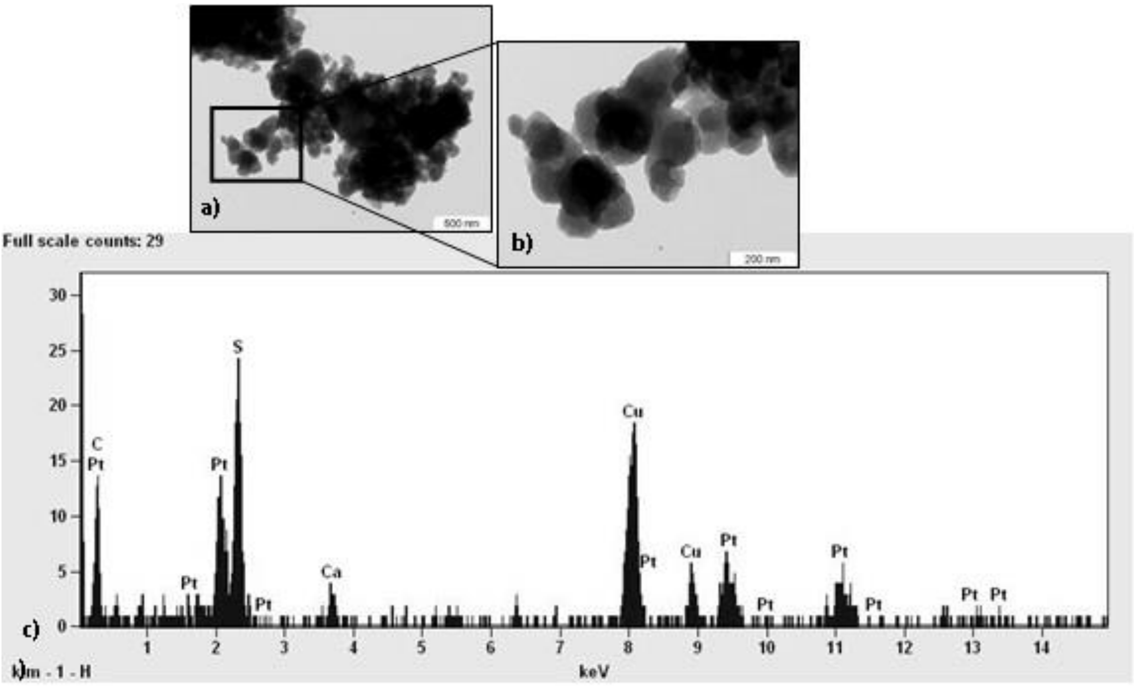

Figure 6-5 - TEM image (a) and b)) and EDS spectrum (c)) of the precipitates obtained in the batch assays in which platinum loaded stripping phase (seawater) was added to the metabolic products.

Some PtS_2 precipitates are nanoparticles and others have sizes smaller than 150nm. These results are shown in Figure 6-5a) and b). The EDS analysis confirmed that the particles are composed by Pt and S, reinforcing the hypothesis that PtS_2 was formed (Figure 6-5 c)). Calcium was also identified in the TEM-EDS spectrum. This element was already detected in the SEM-EDS spectrum and, as explained before, comes from the culture medium and/or from seawater. Carbon and copper identified in the TEM-EDS spectrum are from the supporting grid, since those elements were also present in the background areas. The precipitate obtained in the assay in which palladium loaded stripping phase (seawater) was added to the metabolic products solution was analysed by SEM-EDS and the results are presented in Figure 6-6.

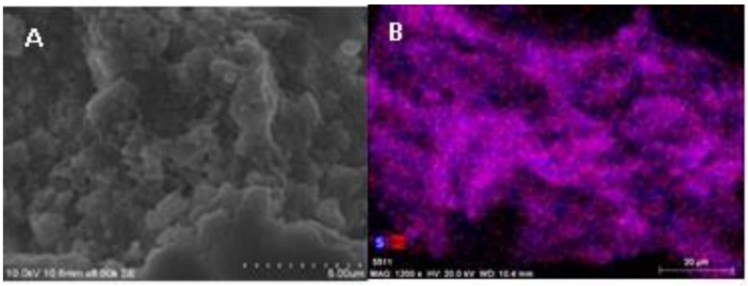

Figure 6-6 - SEM image (A) and XEDS mapping (B) of the precipitates obtained in the batch assays in which palladium loaded stripping phase (seawater) was added to the metabolic products.

The result obtained by SEM, Figure 6-6A, indicates that the precipitates are agglomerates of particles with a spherical morphology. The XEDS mapping micrograph, Figure 6-6B, showed an overlap of the elements Pd and S.

In the SEM-EDS analysis (Figure S9 in the Appendix 2) the peaks with the highest intensity were assigned to Pd and S elements, with an atomic percentage of 6.6% and 21.1%, respectively, which corresponds to an atomic ratio around 3, higher than we should expect for PdS. This ratio can result from the excess of sulphide in relation to palladium in the mixed solution (0.38 mmol Pd to 3.5 mmol sulphide), as obtained before for platinum precipitates. The EDS analysis showed the presence of other elements, namely Fe, Na, Mg, Cl, K and Ca from the culture medium and/or from seawater, as happened before with the platinum precipitate. The results obtained with SEM-EDS were confirmed and supplemented by TEM-EDS analysis (Figure 6-7a)-c)), proving that the particles are mostly clustered and present a spherical morphology with sizes between 70 and 140 nm (Figure 6-7 a) and b)).

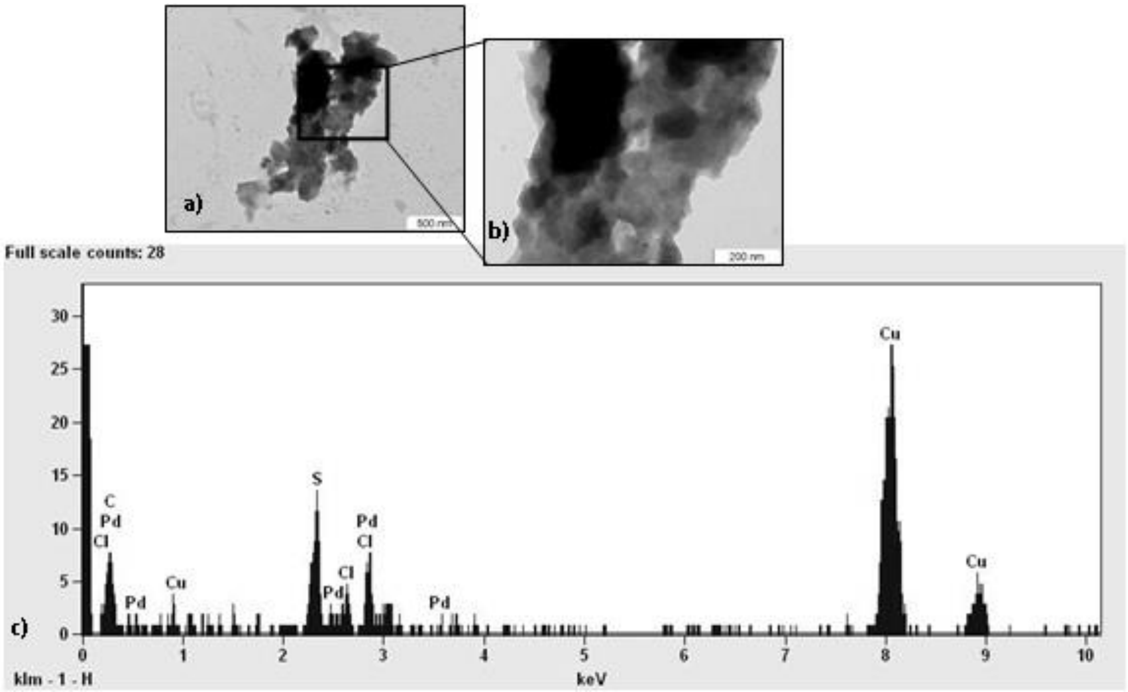

Figure 6-7 - TEM image (a and b) and EDS spectrum (c) of the precipitates obtained in the batch assays in which palladium loaded stripping phase (seawater) was added to the metabolic products.

The results of TEM-EDS analysis (Figure 6-7c)) also corroborate the SEM-EDS results, confirming that Pd and S are the main elements of the precipitate, which is consistent with the formation of PdS. The chlorine element, already detected in the SEM-EDS spectrum, was again identified in TEM-EDS spectrum and it comes from the nutrient medium and/or from seawater. Carbon and copper identified in the TEM-EDS spectrum are from the supporting grid, since they were also present in the background areas. According to the literature, the synthesis of many metal sulphides is well explored due to their potential applications. Joo and colleagues (2003) reported the synthesis of MnS, PbS, ZnS, and CdS nanocrystals through the reaction of metal oleylamine complexes with sulphur elements. Li and colleagues (1999) synthesized uniform nanoparticles of Ag_2S, CdS, MnS, PbS, ZnS and noble metals under hydrothermal or atmospheric conditions with the aid of linoleic acid and other fatty acids (Ng *et al.*, 2006). Although the synthesis of PGM sulphides is less studied, some examples of chemical methods developed aiming PdS formation are reported, such as the formation of polycrystalline PdS thin films as a good alternative for solar applications (Ferrer *et al.*, 2007), the preparation of colloid PdS nanoparticles by solvated metal atom dispersion (SMAD) (Jose and Jagirdar, 2010) and, more recently, Zhang and colleagues (2012) mentioned a novel egg-shell Pd–S catalyst prepared by sulphidizing Pd/C with H_2S and the egg-shell Pd–S/C, which demonstrated to be an efficient hydrogenation catalyst. The use of biological methods aiming at PGM biorecovery seems particularly promising since these methods have some advantages compared to the chemical ones, as they are cleaner and more attractive from an economic point of view, and particularly suitable for metals recovery at low concentrations (Macaskie *et al.*, 2010). Most of the studies, even those performed using SRB, report the formation of metallic Pd (eg., Yong *et al.*, 2002; Baxter-Plant *et al.*, 2003) and Pt (eg, Rashamuse and Whiteley, 2007) and not metal sulphides as the result of biological recovery. Hence, to our knowledge, the formation and recovery of Pt and Pd sulphides as a result of bacterial interaction is poorly mentioned in the literature.

After a literature survey, several applications of PdS and PtS_2 were find. PdS is known as a semi-conductor enabling its use in a wide range of catalytic processes, such as hydrodesulfurization (Zirka and Mashkina, 2000; Ermakova et al, 2002; Mashkina and Khairulina, 2002), hydrogenation (Shalimova *et al.*, 1991) and in the selective synthesis of methanol from carbon monoxide and hydrogen (Koizumi *et al.*, 2001). Palladium sulphide has also been used as light image receiving material with silver halides (Kyama and Iwata, 1996; Tanabe, 1991), for lithographic films (Yamamoto *et al.*, 1987) and lithographic plates with high resolution (Tonomura and Handa, 1996; Idota and Yagihara, 1986). On the other hand, platinum sulphide is employed in the hydrogenation of halogen-containing nitro compounds to amines with high selectivity and on dehalogenation (Dovell and Greenfield, 1965; Greenfield and Dovell, 1967) and reductive alkylation (Dovell and Greenfield, 1967; Bergfeld and Zengel, 1980). The combination of both chemical and biological methods, as performed in this work by combining liquid-liquid extraction with the use of bacterial metabolic products can be a promising and economic solution for the recovery Pd and Pt as metal sulphides. In fact, liquidliquid extraction can be a solution for the selective recovery of PGM from pregnant solutions coming from the leaching of PGM-rich wastes, while, subsequently, the application of biological methods can be a viable alternative for the final recovery of those metals, namely as nanoparticles. The present work also demonstrated that seawater, easily available at low-cost, can be a very effective stripping agent, from which Pt and Pd can be further effectively recovered by biological means as metals sulphide nanoparticles or with a size smaller than 150 nm.

4. CONCLUSIONS

The present research demonstrates a solvent extraction application of N,N-dimethylN,N-dicyclohexylsuccinamide (DMDCHSA) for palladium (II) extraction and the potential of seawater as a new stripping agent for Pd(II) and Pt(IV) stripping. The obtained results allowed to conclude that Pt(IV) and Pd(II) can be easily and quantitatively stripped from the loaded N,Ndimethyl-N,N-dicyclohexyltetradecylmalonamide (DMDCHTDMA) and DMDCHSA organic phases, respectively, using seawater, which is an inexpensive and environmental friendly stripping agent. The present work also demonstrates the feasibility of coupling chemical and biological technologies aiming at palladium and platinum recovery. In fact, the mixture of bacterial metabolic products to palladium and platinum loaded stripping phases of seawater obtained from the previous application of liquid-liquid extraction, allowed the recovery of palladium and platinum sulphides nanoparticles or particles with sizes that do not exceed 150 nm, with potential functional applications.

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CHAPTER 7

Recovery of gold (0) nanoparticles from aqueous solution using an effluent from a bioremediation process

ABSTRACT

The use of biological processes aiming the recovery of gold from low concentrated solutions coming from leaching of secondary sources is gaining increasing importance due to the scarcity of the primary resources and to the economic and environmental advantages usually presented by these methods. Thus, the addition, in batch and in continuous, of different solutions containing biogenic sulphide, generated by the activity of sulphate-reducing bacteria, to gold (III) solutions, was investigated for that purpose. In the batch experiments, Au_2S nanoparticles with sizes between 6 and 14 nm where obtained (corresponding to 100% of Au(III) removal from solution) if the biogenic sulphide was generated in a typical nutrient medium for SRB, while Au(0) nanoparticles with sizes below 8 nm, where attained (corresponding to 62% of Au(III) removal) if an effluent from a SRB bioremediation process to treat AMD was used instead. These results stimulated the development of a continuous process of addition in which two sulphide-rich effluents, resulting from a SRB bioremediation process treating two types of AMD (from an uranium mine and from a polysulphide mine) were tested. In both cases Au(0) nanoparticles with sizes between 6 and 15 nm where mainly obtained, being the percentages of Au(III) removal from solution from 76% to 100%. The processes described allow the simultaneously treatment of AMD and the obtainment of metallic gold nanoparticles, a product with high applicability (e.g. in medicine, optical devices and in catalysis) and economic value. The synthesis process described in this work can be considered a novelty, since it is the first time, to our knowledge, that the use of an effluent from a SRB bioremediation process is reported for gold (III) recovery as gold (0) nanoparticles.

Keywords: Gold, Nanoparticles, Bioremediation, Acid Mine Drainage, Sulphate-Reducing Bacteria

Assunção, A., Vieira, B., Lourenço, J.P., Costa, M.C. Recovery of gold (0) nanoparticles from aqueous solutions using effluents from a bioremediation process. (*submitted*)*

*This scientific paper is based on an International and National Patent Pending

1. INTRODUCTION

Gold, a precious metal with singular beauty due to its colour, has been historically a subject of great interest due to its characteristics, which include excellent electrical conductivity, low contact electrical resistance, outstanding corrosion resistance and low toxicity. These unique characteristics confer on it specific applications (Syed, 2012). Nowadays, gold is used for several purposes such as jewellery, high-tech industries, medical applications and electronic and electrical industries (Ramesh *et al.*, 2008; Spitzer and Rodnei, 2004; Baba, 1987). More recently, the use of gold nanoparticles (NPs) was extended to recognition DNA, hereditary medicine in hyperthermia of tumour, optical coatings, scanning tunnelling microscopes as conductive tips, as well as in catalysis (Cai *et al.*, 2011; Binupriya *et al.*, 2010; Shankar *et al.*, 2005).

Due to its limited availability in nature, gold recovery from both primary and secondary sources is becoming increasingly important (Konishi *et al.*, 2006). Therefore, its recovery from aqueous solutions namely, those resulting from leaching of gold-containing wastes, has been subject of wide investigation. Several processes are described in literature and some of them report gold recovery as Au(0) NPs (He *et al.*, 2007; Mata *et al.*, 2009; Binupriya *et al.*, 2010), which can be advantageous due to their wide range of applications resulting from specific properties arising from their nanoscale. According to Cai and co-workers (2011) the biosynthesis of metal NPs usually presents high selectivity and can operate at room temperature and pressure, without requiring the reagents addition.

The physical and chemical conventional methods used in the synthesis of gold NPs are frequently toxic to the environment due to the use of toxic reagents, or due to the use of extreme temperature and pressure conditions, which increases the complexity and costs of the processes. Therefore, the interest in building "green" technologies for the synthesis of gold NPs has also increased, and consequently the interest and development of biological processes has attracted special attention. For the biosynthesis of gold NPs many biological systems have been proposed, such as, systems based on the use of fungi (Bhainsa and Souza, 2006; Senapati *et al.*, 2005; Ahmad *et al.*, 2003a), algae (Konish *et al.*, 2004; Singaravelu *et al.*, 2007; Mata *et al.*, 2009), bacteria (He *et al.*, 2007; Shahverdi *et al.*, 2007) and plants (Sathishkumar *et al.*, 2009a; Sathishkumar *et al.*, 2009b; Shankar *et al.*, 2003). For example, *Shewanella algae* was able to reduce Au(III) ions with the assistance of hydrogen gas, forming extracellular gold NPs with a size between 10–20 nm (Konish *et al.*, 2004). The fungi *Verticillium* sp (Mukherjee *et al.*, 2001), *Fusarium oxysporum* (Mukherjee *et al.*, 2002) and the actinomycete *Thermomonospora* sp (Ahmad *et al.*, 2003b) and *Rhodococcus* sp (Ahmad *et al.*, 2003c) were also used to synthesize NPs intra- or extracellularly. Recently, investigations on *Rhodopseudomonas capsulata* was conducted on the reduction of Au(III) ions forming gold nanoplates (He *et al.*, 2007). The synthesis of gold NPs may occur inside or outside the cells, however, being preferable the extracellular synthesis, since it simplifies the downstream process, making it more efficient and consequently less expensive, aiming the applications of the obtained particles.

Sulphate-reducing bacteria (SRB) have been described as having bioremediation potential for metals removal during downstream processing from a range of industrial plants (Hao, 2000). Once under anaerobic conditions, SRB reduce sulphate to sulphide, which leads to the formation of metal sulphide precipitates (Chang *et al.*, 2000). Nevertheless, SRB are used successfully in processes that involve the removal of metals and sulphate from Acid Mine Drainage (AMD) (Kaksonen *et al.*, 2003). The metal sulphide precipitation has many advantages, including the production of lower sludge volumes and lower solubility products, comparing to hydroxide precipitation. Moreover, it is possible to recover the metals from the sulphide sludge (Hao, 2000). Bhagat and colleagues (2004) demonstrated the use of sulphide generated by SRB for the removal of Au(III), Cu(II) and Fe(III) from aqueous solutions and the obtained precipitates were mostly metal sulphides, namely gold (I) sulphide.

The traditional processes for AMD remediation, based on the chemical neutralization and hydroxides precipitation, have some disadvantages, such as, high cost, low efficiency in sulphate's remediation and the formation of large sludge volumes, which requires a process for disposal (Chatterjee and Dasgupta, 2005). In alternative, bioremediation processes have been developed, particularly using SRB (Costa and Duarte, 2005; Diels *et al.*, 2002; U.S. Pat. nº 6,852,305). The SRB in anaerobic conditions has the ability to oxidize simple organic compounds, using sulphate as an electron acceptor, reducing it to sulphide (Postgate, 1984). The contact of the metal ions dissolved, for example, iron, copper and zinc, with the biological generated sulphide, causes the metals precipitation, as metal sulphides (Bratcova et al, 2002; Costa and Duarte, 2005; Cabrera *et al.*, 2006; Vitor *et al.*, 2015).

Recently, Vitor and colleagues (2015) have used an effluent containing sulphide resulting from a SRB bioremediation process for AMD treatment, for the synthesis of several metal sulphides and respective TiO_2 nanocomposites, in particular, ZnS and ZnS/TiO_2.

In the present paper the recovery of Au(0) NPs from gold (III) aqueous media by the addition of several solutions containing biologically generated sulphide by SRB, including effluents from the bioremediation process reported by Vitor and colleagues (2005), is described.

2. EXPERIMENTAL

2.1. Batch Experiments for gold recovery

The batch experiments for gold recovery using filtrated supernatant from SRB growth in nutrient medium (Experiment **A1**) and using bioreactor's effluent from a SRB bioremediation process for AMD treatment (Experiment **A2**) are represented in Figure 7-1.

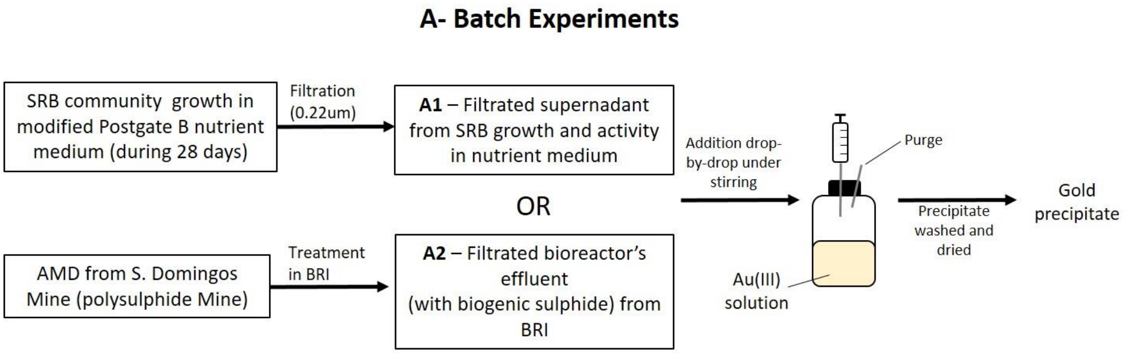

Figure 7-1 - Scheme of the batch experiments aiming gold removal/recovery from aqueous medium. Sequential steps for gold recovery, in a batch system, using filtrated supernatant from SRB growth in modified Postgate B nutrient medium (Experiment **A1**) or using bioreactor's effluent from a SRB bioremediation process for S. Domingos mine AMD treatment, from Bioreactor I (BRI) (Experiment **A2**).

2.1.1. Using the Supernatant from the batch growth of SBR in nutrient medium

A SRB community enriched from the wetland of Urgeiriça mine (North of Portugal), grown and maintained in sub-cultures originally containing species mainly affiliated to *Desulfovibrio desulfuricans* (Martins *et al.*, 2010) was used as inoculum. This culture was grown and maintained in modified Postgate B medium (Postgate, 1984) with the following composition: 0.5 g L^{-1} $K_3PO_4.H_2O$; 1 g L^{-1} NH_4Cl; 1 g L^{-1} $CaSO_4.2H_2O$; 1 g L^{-1} yeast extract; 2 g L^{-1} $MgSO_4.7H_2O$; 0.01 g L^{-1} resazurin; 0.5 g L^{-1} Na_2SO_3; 7.75 g L^{-1} $C_3H_5NaO_3$) in 120 mL glass bottles, at room temperature (21 ± 1 °C) in anaerobic conditions, achieved by purging the medium with nitrogen gas and 10 mL of sterile liquid paraffin, was added. Throughout the experiments, pH, redox potential (Eh), sulphate and sulphide concentrations were determined weekly in order to monitor the bacterial growth. After 28 days of growth, a 30 mL sample was collected, centrifuged at 4000 rpm for 10 min and filtered on a 0.22 μm filter (syringe filter, Puradisc FP 30, Whatman) to avoid the presence of bacteria and obtain a solution resulting from the bacterial growth.

25 mL of a gold (III) solution (Sigma-Aldrich, Gold atomic spectroscopy standard concentrate, 1.00 g L^{-1} Au standard solution) with gold concentration of 67 mg L^{-1} was added in 30 mL glass bottles. The bottles were then sealed with butyl rubber stoppers and aluminum seals. 5 mL of the filtrate solution from the bacterial growth was added to the gold (III) solution under stirring, drop-by-drop, using a syringe.

pH, Eh, sulphate and sulphide concentrations were measured both in the filtrate and in the samples of the mixture collected after 1, 3 and 7 days of contact. The concentration of gold (III) was also analyzed.

All assays were performed with four replicas and were carried out under sterile conditions. Figure 7-1–**A1**, represents a scheme of this batch experiment.

In order to understand the role played by sulphide in gold (III) removal, a similar assay was made using a chemical sulphide solution (Na_2SO_3, Panreac) instead of the filtrate obtained after bacterial growth. The used sulphide solution had 200 mg L^{-1} of sulphide, a similar concentration to the one present in the filtrate resulting from SRB growth. That experiment was performed in duplicate under sterile conditions.

2.1.2. Using a bioreactor's effluent from a SRB bioremediation process for AMD treatment

In these experiments, the gold (III) solution with a gold concentration of 180 mg L^{-1} was placed in 120 mL glass bottles. The bottles were capped with butyl rubber stoppers and a needle was placed in the rubber to relieve the pressure. The bioreactor's effluent (from BRI) was collected, filtered on a 0.22 μm filter (syringe filter, Puradisc FP 30, Whatman) and added drop-by-drop with the syringe to the gold (III) solution under stirring, as shown in Figure 7-1-**A2**.

In this assay, the volume of the added bioreactor's effluent was selected in order to have a molar ratio between sulphide and gold (III) of 1.5. The batch assays were carried out in duplicate, at room temperature, using sterilized material through autoclaving.

The filtrated bioreactor's effluent parameters, as pH, Eh, sulphate and sulphide concentrations, were analyzed and the results are showed in Table 7-1. After the addition of the bioreactor's effluent to the gold (III) solution, samples were collected at different times: after 1 hour and after 1, 3 and 7 contact days. In these samples the pH, Eh, sulphate and sulphide concentrations and gold (III) concentration were also measured.

Table 7-1 - Parameters of the filtrated bioreactor's effluent (BRI) before addition to the gold (III) solution

Parameters	**pH**	**Eh (mV)**	**Sulphate concentration (mg/L)**	**Sulphide concentration (mg/L)**
Bioreactor's effluent (BRI)	8.09	-395	44	156

2.2. Continuous Experiments for gold recovery

Effluents from a bioremediation process for AMD treatment were used to recover gold (III) from an aqueous solution, in a continuous system. The effluent from Bioreactor I (BRI) was obtained in bioremediation's treatment of AMD from S. Domingos' Mine (Experiment **B1**), while the effluent from Bioreactor II (BRII) was obtained in the treatment of AMD from Quinta do Bispo's Mine (Experiment **B2**). Figure 7-2 shows a scheme of the continuous experiments.

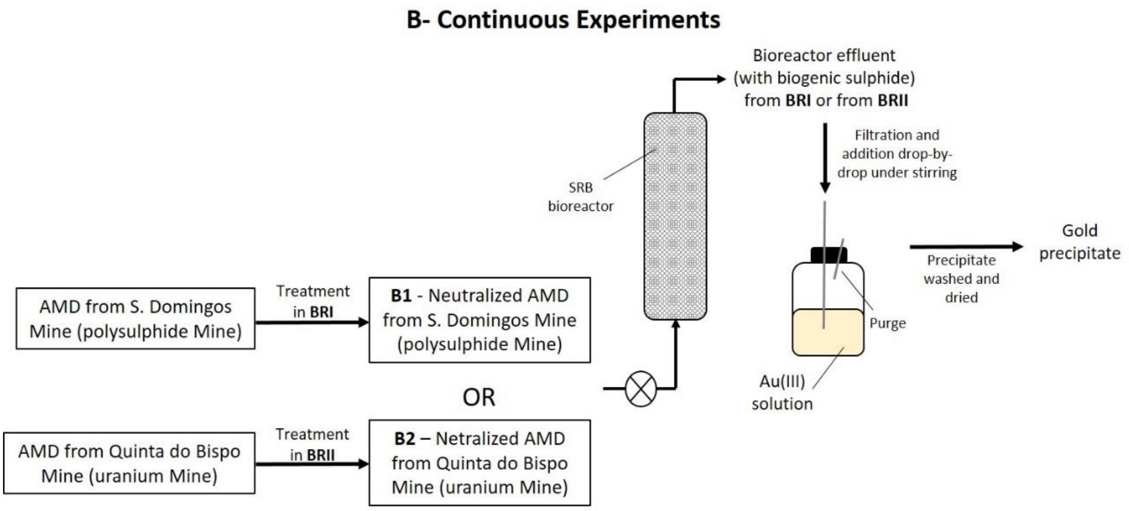

Figure 7-2 - Scheme of the continuous experiments aiming gold removal/recovery from aqueous medium. Sequential steps for gold recovery, in a continuous system, using two different bioreactor’s effluents, one from the treatment of an AMD originated from a polysulphide mine (Experiment **B1**) and the other one from the treatment of an AMD coming from an uranium mine (Experiment **B2**).

In the continuous system, aiming at gold recovery, the gold (III) solution was coupled downstream to the bioremediation process. In this system described in Figure 7-2, two bioreactor’s effluents were tested, one from the treatment of the AMD from S. Domingos’ mine (BRI) (Figure 7-2- **B1**) and the other from the treatment of an AMD coming from the uranium mine of Quinta do Bispo, located in North Portugal (BRII) (Figure 7-2- **B2**). Both AMD were neutralized with calcite tailing, before entering the bioreactor to ensure a suitable pH for the growth and activity of SRB in the bioreactor, as shown in the first step of Figure 7-2.

Previous experience as shown that a combined process consisting of an anaerobic sulphate-reducing bioreactor, following neutralization with calcite tailing, produces water complying with legal irrigation requirements from synthetic AMD (Vitor *et al.*, 2015).

In all the experiments, the bioreactor’s effluent was added to the gold (III) solution, drop-by-drop, using a syringe under stirring.

2.2.1. Bioreactor’s Effluent characterization

The bioreactor’s effluents produced in this bioremediation process had sulphide generated by the SRB present in the bioreactor. This sulphide is an environmental hazard (Mandal *et al.*, 2006; Labrenz and Banfield, 2004), thus a system was designed to use it, aiming gold recovery from an aqueous solution, under controlled conditions. The bioreactor is an Upflow Anaerobic Packed-Bed Reactor (UAPB) inoculated with a SRB community and the AMD bioremediation processes contain an additional upstream neutralization step, as described by Victor and colleagues. Ethanol was used as carbon source to promote SRB growth and activity and phosphorous and nitrogen were also added regularly.

The BRI was treated AMD from the polysulphide mine of S. Domingos (containing several metals such as Fe, Cu and Zn), while BRII was treating AMD from the uranium mine of Quinta do Bispo (containing metals such as Fe, Al, Mn and U). The effluents of both bioreactors (BRI and BRII) were characterized in terms of pH, Eh and concentrations of sulphate and sulphide and the results are displayed in Table 7-2.

Table 7-2 – Parameters of the filtrated effluents from BRI and BRII before the addition of the gold (III) solution

Parameters	**pH**	**Eh (mV)**	**Sulphate concentration (mg/L)**	**Sulphide concentration (mg/L)**
Bioreactor's I effluent	6.44	-329	26	398
Bioreactor's II effluent	6.83	-292	27	140

The system used for gold recovery consisted in a 1L reaction vessel containing gold (III) solution under stirring (Velp Scientifica ARE magnetic stirrer) in which the bioreactor's effluent was collected drop-by-drop, as demonstrated in Figure 7-2.

In the experiment with the effluent from BRI, the initial Au(III) concentration in metal solution was 100 mg L^{-1}, while in the experiment with effluent from BRII, the Au(III) concentration was 200 mg/L and in both cases the gold (III) solutions were deaerated in an ultrasound bath (J.P. Selecta Ultrasons-H) for 15 min.

The possible contamination of the gold precipitate with bacteria and/or metals precipitates from the bioreactor was avoided by using a filter system placed immediately before the reaction vessel. That filter was composed by a coarse glass wool pre-filter in a glass column (14 cm long, 2 cm diameter), followed by a 0.2 µm syringe filter (Whatman Puradisc 25 AS), as described by Vitor and colleagues (2015). The volume of the bioreactor's effluent was added until a molar ratio of 1.5 between sulphide (from the effluent) and gold (III) was achieved. After the effluent's addition the parameters of the mixture were measured (pH, redox potential, sulphate and sulphide concentrations and gold concentration) and the precipitate was characterized.

2.3. Analytical methods

In order to monitor the growth of the SRB community, samples from the batch cultures were periodically collected using a sterile syringe and the optical density at 600 nm (OD_{600}) was measured in each sample. After centrifugation at 4000 rpm for 5 min, redox potential and pH were determined using a pH/E Meter (GLP 21, Crison) and the sulphate concentration was quantified by UV-visible spectrophotometry (Hach-Lange DR2800 spectrometer) using the method of SulfaVer®4.

As mentioned above, in the batch experiments, samples were periodically collected, while in the continuous experiments the samples were collected at the end of the bioreactor's effluent addition.

In the mixtures containing the gold (III) solution and the sulphide solution, the sulphide concentration was measured immediately after sampling, using an UV–Visible spectrophotometer (DR 2800, Hach-Lange) by the Methylene Blue Method (665 nm, Hach-Lange). After centrifugation at 4000 rpm for 5 min, redox potential, pH and sulphate concentration were also measured using the same methodology and equipment described above. The gold (III) concentration was analysed using the thiamine-phloxine method described by Fujita and colleagues (1999). In an assay tube with 1mL of the test solution, 0.5 mL of 0.5% (w/v) methylcellulose, 1 mL of 10 mM thiamine, 2 mL of EDTA-citrate buffer (0.05 M EDTA, 0.1Mcitric acid pH 4.6), 1 mL of 1 mM phloxine B (Sigma–Aldrich Chemical Company) and 4.5 mL of distilled water were added. The tubes were incubated in a water bath (20 min, 40°C) and the absorbance was measured at 570 nm after cooling. The blank was prepared the same way as the samples by replacing the gold sample by distilled water (Deplanche and Macaskie, 2008).

2.4. Precipitates characterization

The precipitates obtained in the different experiments were characterized. In order to recover the precipitates, samples of the mixture were collected, centrifuged at 4000 rpm for 10 min and the pellets were washed 3 times with ethanol 70% and the precipitates were then dried in vacuum (Binder, VDL) at 37°C ± 1°C.

The obtained precipitates were analysed by X-Ray Diffraction (XRD) using a PANalytical X'Pert Pro powder diffractometer, operating at 45 kV and 35 mA, with Cu Kα radiation filtered by Ni. The XRD patterns were recorded using an X'Celerator detector, with a step size (2θ) of 0.03°, and a time per step of 1000 s. Peak analysis and crystalline phase identification were conducted using the High-Score Plus software, with the ICDD PDF-2 database.

Transmission Electron Microscopy (TEM) (Hitachi, H8100 model, with a LaB6 filament) coupled to Energy Dispersive X-ray Spectroscopy (EDS or EDX) (ThermoNoran) were used for the determination of the particles size, morphology and elemental composition.

3. RESULTS AND DISCUSSION

Metals are usually toxic for microorganisms, such as SRB, since essential ions can be substituted on cellular sites causing functional groups blockage of important molecules such as enzymes (Martins *et al.*, 2009). Therefore, in the present work solutions resulting from SRB growth in several conditions were added to Au(III) solutions, instead of the direct addition of gold (III) to the nutrient medium during bacterial growth. The advantage of the present methodology is the fact that it does not require the use of metal-resistant bacteria. A solution resulting from SRB growth and two effluents from a SRB bioremediation process described by Victor and colleagues (2015) for AMD treatment were used for that purpose. As described in Figure 7-1 the experiments were carried out in batch (Experiments **A1** and **A2**) and in a continuous system (Experiments **B1** and **B2**).

3.1. Batch experiments for gold recovery

The batch experiments for gold recovery were performed using the filtrated solution resulting from SRB growth in batch, in nutrient medium (Experiment A1) and the bioreactor's effluent collected from BRI (Experiments A2).

The filtrated solution used in experiment A1 was obtained after SRB growth for 28 days. After that time around 55% of sulphate was consumed, producing 247 mg L^{-1} of sulphide. The optical density (600 nm) of the solution reached 0.6, due to high bacterial growth. The molar ratio between gold (III) and sulphide in the experiment was 1:1.5.

The A1 experiment showed that in less than one hour all the Au(III) was removed from the solution and a dark precipitate was formed. This result was followed by the removal of 93% of sulphide, which may indicate that the sulphide present in the filtrated solution might be responsible for the Au(III) removal. In order to prove this hypothesis, an assay using a “chemical” sulphide solution with a sulphide concentration of 200 mg L^{-1} was performed in the same batch conditions. The results are shown in Figure 7-3 A and demonstrate that sulphide seems to be responsible for gold precipitation.

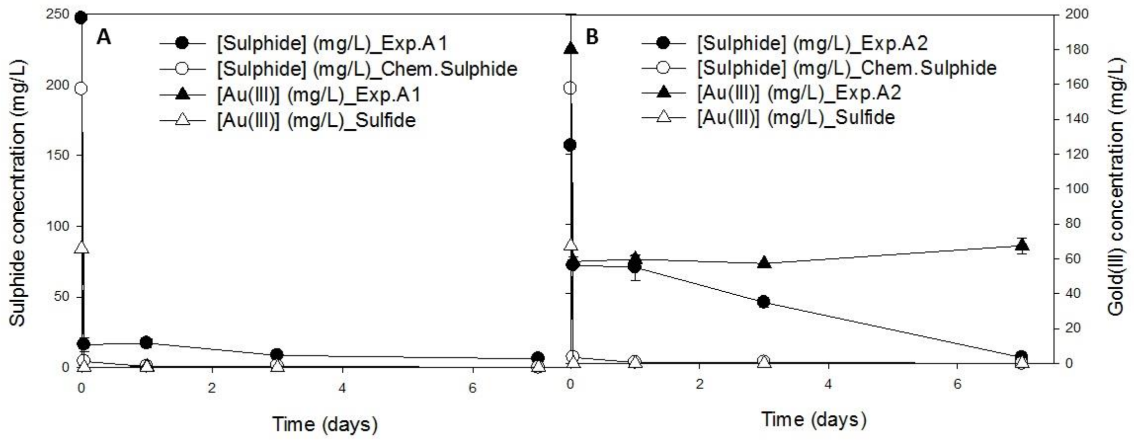

Figure 7-3 – Evolution of sulphide and gold (III) concentration in Experiment **A1** (A) and Experiment **A2** (B), throughout the time. In A and B graphs, the white symbols represent the chemical control with chemical sulphide (white circle) and gold (III) concentrations (white triangle). The black symbols represent the biogenic sulphide concentration (black circle) and the gold (III) concentration (black triangle). Data are the average of four replicates in Experiment **A1** and **A2**, while in assays with the chemical sulphide the data are the average of duplicates. The error bars indicate the standard deviations of the average values (in some cases error bars are smaller than symbols, therefore not visible).

In experiment A2, a brown precipitate was formed. The removal of gold (III) did not exceed 62%, as shown in Figure 7-3 B, while, in assay A1 the removal was complete (100%) and almost immediate. The different percentage of Au(III) removal from the solution and the different colour of the precipitate obtained can indicate that different gold precipitates were formed. Eventually, these results could be associated to the different molar ratio between sulphide and gold (III) used in each experiment, or to the different composition of the biologically generated sulphide solution, considering that one resulted from SRB growth in nutrient medium (A1) and the other from the treatment of AMD (A2). In experiment A1 the ratio between sulphide and gold was 15, thus there were more sulphide species available to interact with gold (III), when compared with experiment A2, in which the ratio was 10 times lower (1.5).

Furthermore, a longer contact time between the metal ion and sulphide does not influence Au(III) removal, since in experiment A1, Au(III) removal within the first hour remained constant independently of the presence of sulphide in the solution, as shown in Figure 7-3 B. Therefore, the interaction between the sulphide and gold (III) ions occurs within the first hour. The decrease of sulphide during the rest of the assay can be related to H_2S volatility, since whenever a sample was collected the sulphide concentration in the solution decreased.

In both experiments the pH also decreased after the addition of the biogenic sulphide to the gold (III) solution. However, in experiment A1 a higher pH decrease was achieved (8.47 to 2.50), while, in experiment A2 the pH decreased from 8.09 to 5.58. The initial pH of the gold (III) solution used in both experiments varied between 1.76 and 1.95, which contributes for the pH decrease in the mixture. The pH decrease can also be related with the dissociation of H_2S, as the sulphide reacts with gold (III) ions (Bhagat *et al.*, 2004).

3.1.1. Precipitates characterization

The precipitates from experiments A1 and A2 were washed and dried for subsequent characterization by XRD and TEM-EDS, in order to determine the crystallography of the precipitate and the particles morphology and size, respectively.

The XRD diffractograms of the obtained gold precipitates are shown in Figure 7-4.

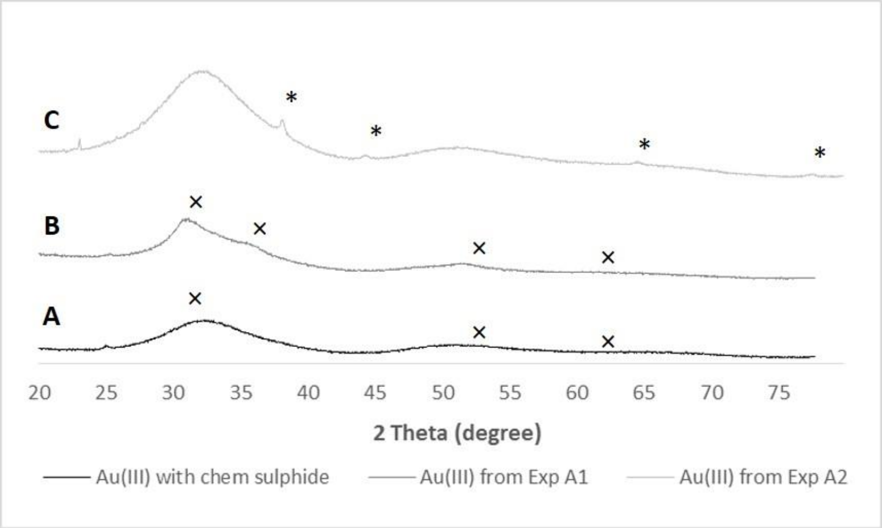

Figure 7-4 - X-Ray powder diffraction patterns for precipitates generated in batch assays: (**A**) Precipitate obtained using a “chemical” solution of sulphide; (**B**) precipitate obtained in experiment A1; (**C**) precipitate obtained in experiment A2.

The XRD analysis corresponding to (A) and (B) diffractograms were not totally conclusive. Some broad peaks (identified as (x)) may correspond to Au_2S (ICDD PDF2 # 01-085-1997). These non-conclusive results of XRD can be associated to the very small size of the particles, which is consistent with the subsequent results of Transmission Electron Microscopy (TEM).

The XRD diffractogram shown in Figure 7-4 C, is more conclusive, since the peaks (identified as (*) in the diffractogram) indicate the presence of metallic gold particles, which could be indexed on the basis of the cubic gold structure (ICDD PDF2 # 01-071-4615). The first peak, not identified in the diffractofram is a typical sulphur peak, which indicates that some elemental sulphur is also present in the sample.

A TEM-EDS analysis of the same precipitates was also performed. The TEM characterization of the precipitate obtained in experiment A1, Figure 7-5-**A1** A and B, confirms that the particles are NPs with very small sizes, between 6-14 nm, which may explain the results of XRD analysis. In addition, those NPs are present in agglomerates. The Energy Dispersive X-ray (EDS) spectrum only shows peaks of the elements Au and S, Figure 7-5-**A1** C, which are consistent with the formation of gold sulphide NPs.

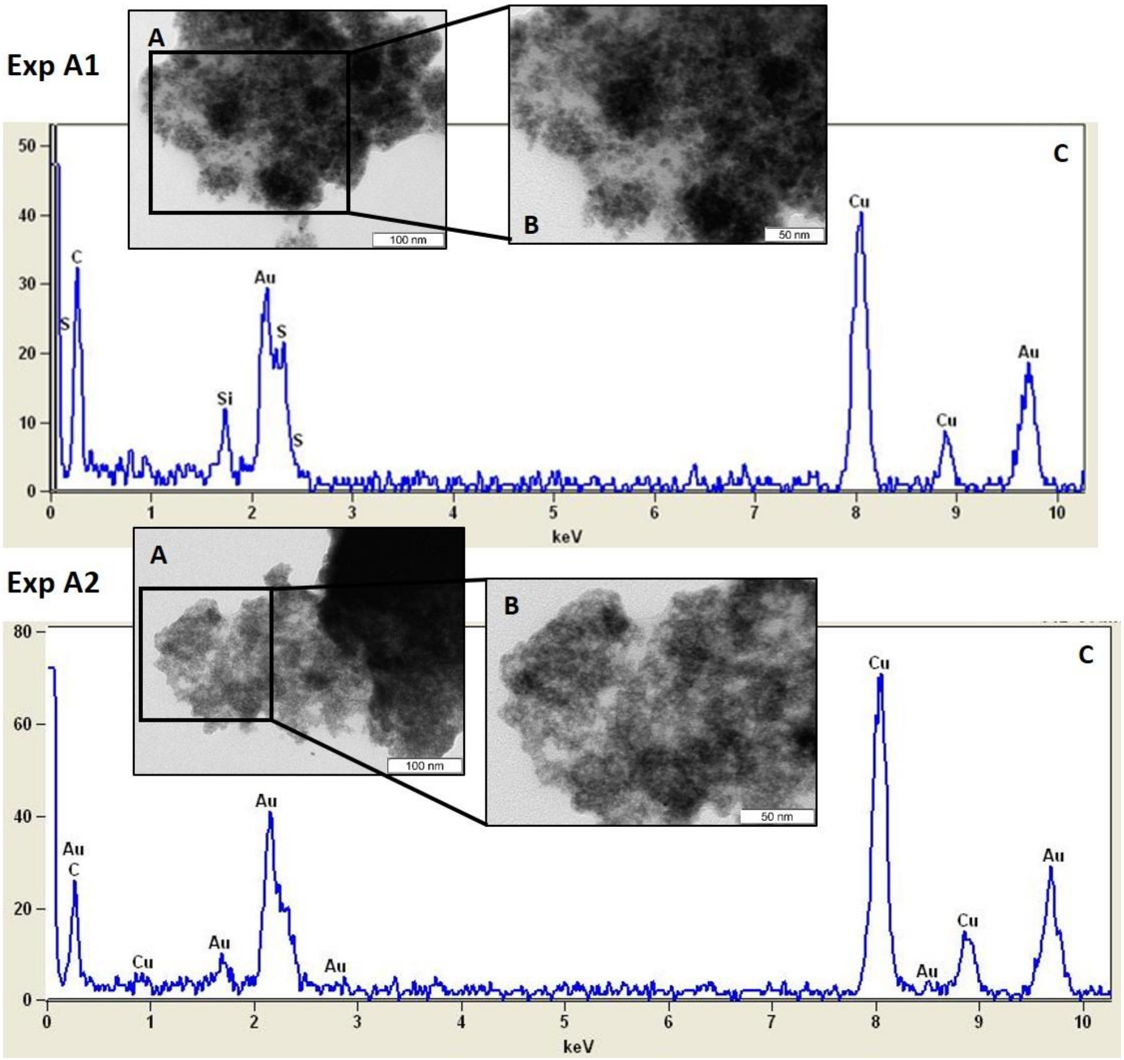

Figure 7-5 - TEM images and corresponding EDS spectrum of the precipitates obtained in Experiments **A1** and **A2**.

In the EDS, the element percentage of sulphur and gold was 69% and 31%, respectively. The higher percentage of the element S in comparison to Au can be justified by the excess of sulphur in the precipitate, thus not all sulphur react with Au to produce Au_2S,but some elemental sulphur may also had been formed. TEM-EDS results of the obtained precipitates, in experiment A2, are shown in Figure 7-5-**A2**. The precipitates were mainly composed by spherical gold NPs agglomerated with a size smaller than 8 nm (Figure 7-5-**A2** A and B). The EDS spectrum, Figure 7-5 C, shows the presence of Au. The Cu element also identified in both EDS spectrum belongs to the sample grid.

In order to understand if it would be possible to obtain similar results in a continuous system, using effluents produced in an AMD bioremediation process, the replace of the batch system for the continuous system was carried out. For that purpose, effluents from two different AMD bioremediation processes were tested, downstream to the bioreactor.

3.2. Continuous system for gold recovery

The experiments developed in a continuous system for Au(III) recovery were performed using bioreactor's effluents from two AMD bioremediation processes. The main difference between these two effluents was the treated AMD and the initial concentration of the gold (III) in the solution. BRI treats AMD from S. Domingos' mine while Bioreactor II treats AMD from the uranium Quinta do Bispo's mine.

Although, two different gold (III) concentrations, were used, the molar ratio between sulphide (from the effluent) and gold (III) was maintained at 1.5, since in the batch assays it was demonstrated that this is the most suitable ratio to obtain gold (III) removal percentages up to 60% as metallic gold NPs. In the continuous assays, the time required to achieve this ratio depends on the AMD flowrate. The flowrate in these bioreactors ranged from 2.5 to 5.0 mL/h and the experiments lasted no more than 25 hours.

Sequential pictures of the continuous system aiming gold recovery, using bioreactor's effluent from AMD bioremediation process, are shown in Figure 7-6.

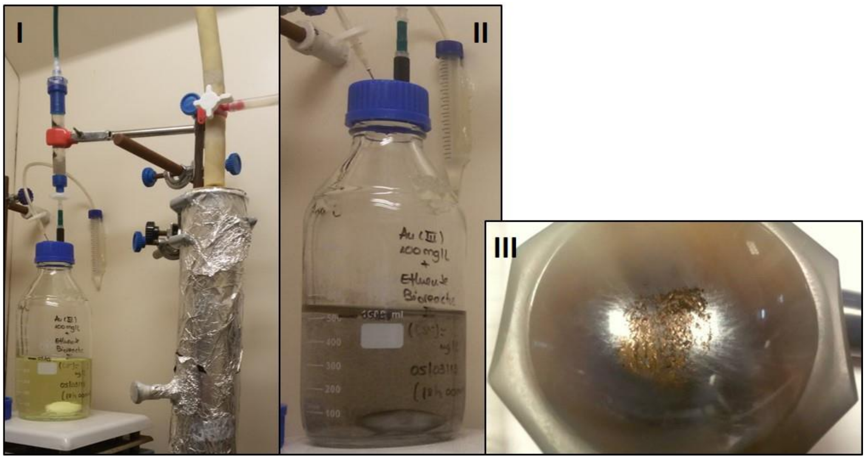

Figure 7-6 – Pictures of the: **I** - Au(III) recovery coupled to the bioremediation system - flask containing the Au(III) solution before addition of the bioreactor's effluent; **II** - mixture of Au(III) solution with the bioreactor's effluent during the addition of the bioreactor's effluents; **III** - obtained gold precipitate.

The pH profile was similar to the one obtained in batch assays. Thus, in both continuous experiments, B1 and B2, the pH decreased from values 6.44 and

6.83 to 2.36 and 2.52 values, respectively. As mentioned above, this pH decrease is associated to the addition of the gold (III) solution with a pH lower than 2.

When the bioreactor's effluent started to be added, drop-by-drop, to the gold (III) solution (Figure 7-6 **I**), the formation of a brown and bright precipitate was observed (Figure 7-6 **II** and **III**). The sulphide from both effluents is probably able to reduce Au(III) into insoluble Au(0) (Figure 7-6 **II** and **III**), which is consistent with the result obtained in the batch assays when the bioreactor's effluent was added to Au(III) solution.

Figure 7-7 contains the percentages of gold (III) and sulphide removal from the solution obtained in both continuous assays.

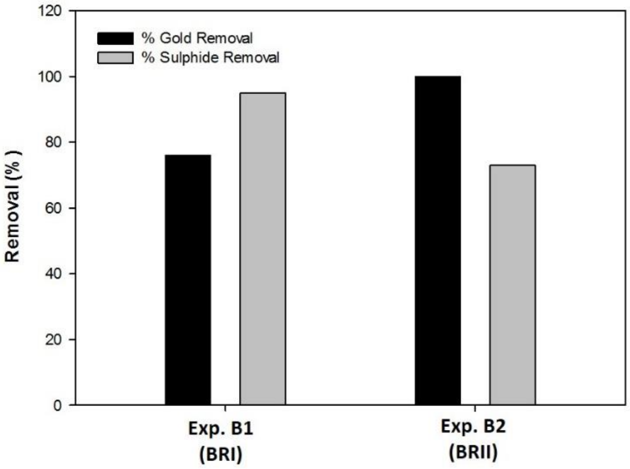

Figure 7-7 – Percentage of gold (III) (in grey) and sulphide (in black) removal for both continuous system (Experiments **B1** and **B2**).

In experiment B1, gold (III) and sulphide removal achieved values of 76% and 95%, respectively, while in experiment B2 the removal of gold (III) and sulphide reached 100% and 73%, respectively.

The results showed that gold removal percentages higher than 76% can be achieved regardless of the effluent used. However, the percentage of gold recovery is probably dependent on the effluent composition.

3.2.1. Precipitate's characterization

The precipitates obtained in the continuous experiments (B1 and B2) were washed, dried and characterized by XRD and TEM-EDS.

Despite the different percentages of gold removal, 76% and 100%, using the bioreactor's effluents, from BRI and BRII, respectively, the characterization of the precipitates has shown to be very similar.

The XRD analysis of the precipitates obtained in experiments B1 and B2 are presented in Figure 7-8.

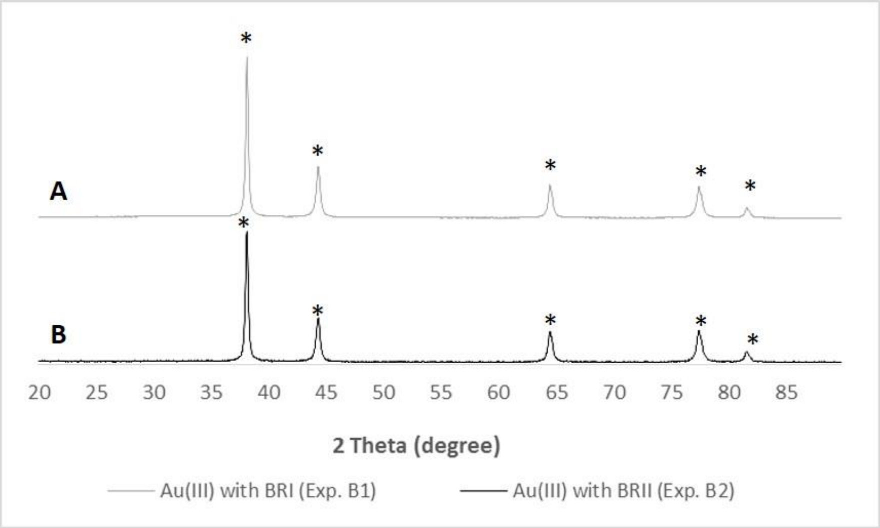

Figure 7-8 - X-Ray powder diffraction patterns of the precipitates generated in the continuous assays using the effluent from the BRI - Experiment **B1** (A) and from BRII -Experiment **B2** (B).

A similar XRD pattern was obtained for both precipitates and the results are consistent with Au(0) particles, which could be indexed on the basis of the cubic gold structure (ICDD PDF2 # 01-071-4615), as shown in the batch experiments with this bioreactor's effluent (Experiment A2), but with more intense and sharp peaks.

XRD pattern revealed intense peaks of Bragg's reflections corresponding to (1, 1, 1), (2, 0, 0), (2, 2, 0), (3, 1, 1) and (2, 2, 2) at 2θ values of 38.2°, 44.5°, 64.7°, 77.7° and 81.2°, respectively. No spurious diffractions, due to crystallographic impurities, were found. The XRD patterns of the samples compared to the standard patterns of gold (0) (represented by '*') with typical peaks for zero-valent gold, confirms that the precipitates are effectively Au(0).

TEM results of the precipitates obtained in experiments B1 and B2 revealed that there are mainly two different shapes, morphology and sizes, as shown in Figure 7-9.

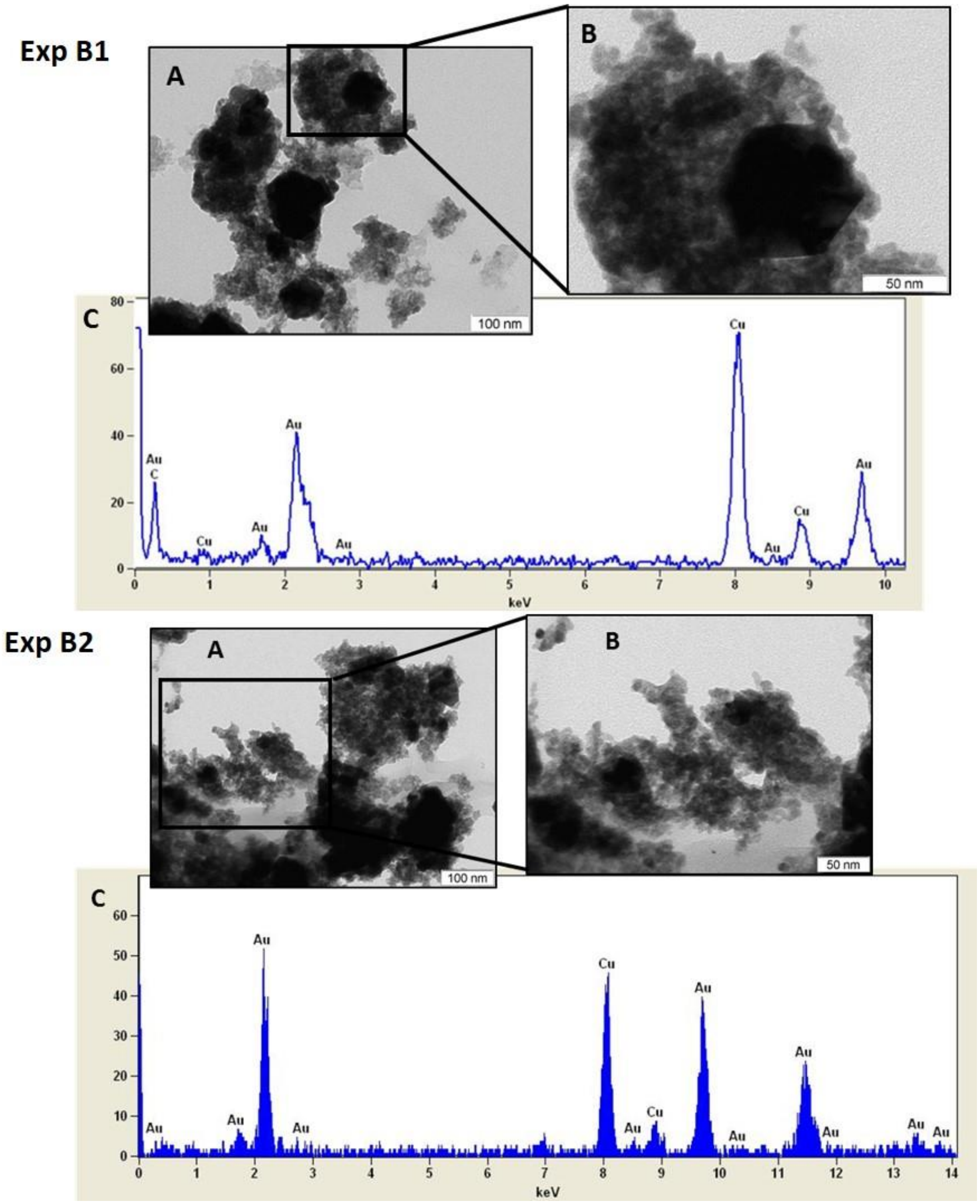

Figure 7-9 - TEM images and corresponding EDS spectrum of the nanosized particles of Au(0) obtained in experiments **B1** and **B2**.

In the experiment B1, the particles with hexagonal morphology are bigger and present a size range of 30-80 nm, while the particles with spherical morphology are smaller, ranging 6-10 nm (Figure 7-9 A and B –**B1**). The particles are mostly found in agglomerates and the EDS analysis identified only peaks corresponding to Au (Figure 7-9 C -**B1**), thus corroborating the XRD results.

A representative TEM image of the obtained precipitates in experiment B2 is represented in Figure 3-1Figure 7-9 A and B -**B2** and reveals a spherical shape of the NPs with a size range of 8–15 nm. The EDS analysis, Figure 7-9 C –**B2**, confirms the result obtained by XRD, once gold was the only element identified in the precipitate. The identification of Cu is due to the composition of the sample grid.

Table 7-3 shows a summary of the results obtained in both systems batch and continuous for each experiment.

Table 7-3 – Summarizing table: Results of gold recovery and particles characterization in each experiment

System	Experiment	Gold removal (%)	XRD	TEM	EDS	Particles Size
Batch	**A1**	100	Cubic Gold Sulphide (Au_2S)	Spherical morphology, Agglomerated	Au and S	6-14 nm
	A2	62	Cubic Gold (Au(0))	Spherical morphology, Agglomerated	Au	< 8 nm
Continuous	**B1**	76	Cubic Gold (Au(0))	Spherical and hexagonal morphology, Agglomerated	Au	6-10 nm (spherical); 30-80 nm (hexagonal)
	B2	100	Cubic Gold (Au(0))	Spherical morphology, Agglomerated	Au	8-15 nm

The results obtained in experiment A1 suggest that the sulphide produced during bacterial growth, in nutrient medium, was able to remove gold (III) from aqueous solution, as gold (I) sulphide NPs, meaning that gold (III) was previously reduced to gold (I), which was combined to the biologically generated sulphide present in solution. Therefore, the production of gold (I) sulphide occurred when the conditions to reduce gold (III) to gold (I) were created and whenever biogenic sulphide was available.

A similar study was already performed in which a bioreactor was used operating on a fill-and-draw basis, using Postgate medium as feed to favour the SRB growth. The sulphide produced by SRB community was used to metals (Fe(III), Cu(II) and Au(III)) precipitation by sparging the metals solution with biogenic sulphide gas or by mixing it with sulphide-rich supernatant from a sulphate reducing bioreactor feed with nutrient medium, without the need of biomass. The obtained precipitates are mainly metal sulphides and also contained unidentified complexes which could include nitrates, chlorides and carbonates (Bhagat *et al.*, 2004). For the assays with gold (III), a black gold (I) sulphide precipitate was formed when biogenic sulphide was bubbled through an Au(III) solution according to the eq. (1), described by Vogel (1996):

$$2[AuCl_4]^- + 3H_2S \longrightarrow Au_2S\downarrow + 2S\downarrow + 6H^+ + 8Cl^- \qquad \text{Eq. (1)}$$

As suggested by Bhagat and colleagues (2004), at room temperatures, elemental gold is dispersed with Au_2S.

It is expected that the same reaction is involved in the formation of Au_2S in our batch study, when the filtrated supernatant from SRB growth in nutrient medium (experiment A1) was used. Once this precipitate is heated to 200°C it decomposes to elemental gold (Puddephatt, 1978). However, using the bioreactor's effluent (experiments A2, B1 and B2) elemental gold was obtained directly, without requiring the use of these high temperatures.

In our experiments, where bioreactor`s effluents from AMD treatment were used, Au(0) NPs were obtained instead of Au_2S. We can speculate that the effluents contain compounds able to reduce Au(III) to Au(0) that were not present in the solution resulting from SRB growth in nutrient medium. No Au(III) removal was observed when AMD was added to the Au(III) solution, which may indicate that the reducing compounds resulted from bacterial activity. Indeed, biological reduction of gold (III) to gold (0) has been reported but only in the presence of biomass. Cells of *Magnetospirillum gryphiswaldense* MSR-1 and *Saccharomyces cerevisiae* showed to be able to reduce gold (III) ions to a zero-valent metal and accumulate them on the cell surface through biosorption (Cai *et al.*, 2011; Lin *et al.*, 2005). Lin and colleagues suggested that the biosorption mechanism of Au(III) involves an apparent redox reaction in which the ionic metal is reduced to the metallic form, as we suppose to occur in our case, but without the need of biomass. In the reported biosorption process, the hydroxyl and carboxylate ion groups of biomass played an important role in the Au(III) binding and consequent reduction to Au(0), mainly by the free aldehyde group of the reducing sugars (Lin *et al.*, 2005).

Actually, the reduction of Au(III) to Au(I) was already reported as an intermediated step before the formation of Au(0) in experiments using different biomass, such as alfalfa (Gamez *et al.*, 2000), dealginated brown alga (Romero-Gonzalez *et al.*, 2003) and *Chlorella*, a single-cell green algae (Greene *et al.*, 1986). These authors have proposed a fast reduction of Au(III) to Au(I), while reduction to Au(0) is reported as slow (Mata *et al.*, 2009). In our experiments (A and B), the reduction of Au(III) to Au(I) occurs without biomass as support, and can be performed by chemical compounds from the nutrient medium and/or can be associated to the bacterial activity.

Some authors suggested that $NADH^-$ and NADH-dependent enzymes are important factors in the biosynthesis of metal NPs (Mukherjee *et al.*, 2002; Ahmad *et al.*, 2002; Senapati *et al.*, 2005; Kumar *et al.*, 2007). The potential of Rhodopseudomonas capsulata, one of the most ecological and environmentally important bacteria, commonly existing in natural environment, for Au(III) reduction was already studied. It was demonstrated that this bacteria, able to grow anaerobically, can successfully produce gold NPs of different sizes and shapes. The authors suggested that R. capsulata secrete cofactor $NADH^-$ and NADH dependent enzymes can be responsible for the Au(III) bioreduction to Au(0) with the formation of NPs. Although the Au(III) bioreduction mechanism played by R. capsulate is not well known, it was suggested that gold (III) reduction is initiated by electron transfer from the NADH by NADH-dependent reductase, as electron carrier and then the gold ions are reduced to Au(0) (He *et al.*, 2007). Another possible explanation are the proteins, since they are the primary biomolecules involved in providing the function of Au(III) reduction and in the function of the size and shape controls (Xie *et al.*, 2007).

These hypothesises might explain the achievement of Au(0) when the bioreactor's effluents were used and the achieved of Au_2S when the supernatant from the SRB growth on a nutrient medium was utilised. This can be related to the bacterial community present in experiment A1 and in experiments A2, B1 and B2. The bacterial community in the bioreactor was probably richer in bacteria that secrete cofactor NADH- and NADH dependent enzymes, like R. capsulate, enabling the reduction of Au(III) to Au(0) NPs. Despite all the bacterial community used in the experiments have been enriched to benefit the sulphate-reducing bacterial growth, each one was subjected to different conditions, which can select a different composition of the SRB communities. The SRB community used to obtain the filtrated solution from bacterial growth was grown in culture medium, while the bioreactor’s effluents were obtained from a SRB community previously acclimatized in the bioreactors with culture medium and then subjected to different AMD. This fact certainly changed the initial bacterial community, justifying the eventual production of different compounds during the bacterial growth and that may be involved in gold (III) bioreduction mechanism.

The results obtained in the present work suggest that it is possible to reduce Au(III) to Au(0) NPs using bioreactor's effluents, rich in biogenic sulphide, from AMD bioremediation processes based on the activity of SRB. These results were obtained without the presence of biomass as reported in previous studies. The described process presents some economic advantages, due to the use of a bioreactor's effluent, able to replace chemical processes that use reagents, as $NaBH_4$, citrate and ascorbate (Binupriya *et al.*, 2010) for the removal/recovery of a valuable material such as Au(0). Furthermore, it takes advantage from the use of H_2S, a toxic sub-product of the AMD bioremediation process, thus with additional environmental benefits.

4. CONCLUSION

The present work demonstrates, for the first time to our knowledge, the ability of effluents from a SRB bioremediation process for AMD treatment, containing biogenic sulphide, to efficiently remove gold (III) from solution, allowing its recovery as Au(0) NPs.

The reduction of gold (III) to metallic gold seems to be, not only dependent on the sulphide ions available in the solution, but may also be dependent on the presence of other compounds produced by the bacterial community in the bioreactor. It can also be concluded that the Au(0) particles recovered in these processes are mostly NPs with very small sizes between 6 to 15 nm with a cubic crystalline structure, which confers them unique properties useful for specific applications.

The process described in this paper is an attractive alternative from both environmental and economic points of view to the traditional chemical methods usually utilized for gold recovery: it allows the valorization of an effluent and is performed at room temperature and pressure, thus avoiding the use of extreme conditions and expensive chemicals. Moreover, the possibility to recover gold as metallic NPs is very important taking into account their high market prices and diverse uses. The recent increase of gold share value may be justified by the significant value usually attributed to this metal during international political and economic crises and by its limited availability. Thus we hope the process described in this work could be a promising contribution aiming to facilitated the recovery of gold, namely from secondary sources.

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CHAPTER 8

Green synthesis and characterization of Ag_2S nanoparticles and Ag_2S/TiO_2 nanocomposites aiming radiation based applications

ABSTRACT

This research describes the synthesis of Ag_2S nanoparticles (NPs) with high yield and via a straightforward process, under ambient conditions (temperature and pressure), by adding to an aqueous silver (I) solution, an effluent, containing biogenic sulphide, from an Acid Mine Drainage (AMD) bioremediation process. The process was performed in batch flasks and coupled downstream to a continuous system for AMD treatment based on the use of sulphate-reducing bacteria. According to XRD and electronic microscopy analysis, precipitates obtained were composed of Ag_2S (a*canthite*) NPs. The NPs obtained in the batch system exhibit spherical and spheroidal morphology with sizes between 18-29 nm and 28-54 nm, respectively, while those obtained coupled to the bioremediation process exhibit spheroidal and cylindrical morphology with size ranging between 15-24 nm. The synthesis of Ag_2S/TiO_2 nanocomposites coupled downstream to the continuous AMD bioremediation process system, was successfully performed using the effluent containing biogenic sulphide. The obtained nanoparticles exhibit mostly a spheroidal morphology with sizes between 30-60 nm and the TiO_2 particles become "decorated" with Ag_2S nanophases, which resulted from deposition of the metal sulfide at the substrates surfaces. The Ag_2S obtained presents a band gap value around 1 eV, which makes this semiconductor able to be used as a solid-state electrolyte, presenting both ionic and electronic conduction at room temperature. This bandgap and the large absorption coefficient turns Ag_2S an effective semiconductor material for photovoltaic applications. Furthermore, the Ag_2S NPs obtained present a lower band gap energy compared with TiO_2 (~3.5 eV), allowing the use of visible radiation by the Ag_2S/TiO_2 nanocomposite. In fact, studies on possible radiation applications of these particles are currently underway, but results were not yet completed at the time of thesis submission.

Keywords: Ag_2S, nanoparticles, Ag_2S/TiO_2 nanocomposite, biogenic sulphide, bioremediation process

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1. INTRODUCTION

Nanotechnology has been an area of growing interest, due to the wide range of potential application in optoelectronic and biological fields. For example, the study of semiconductor nanoparticles has been gaining momentum as a result of their optical and electronic properties (Lu *et al.*, 2005). The study of green processes for the synthesis of nanoparticles allowed the development of an important branch of nanotechnology (Raveendran *et al.*, 2006).

Metal nanoparticles (NPs), particularly silver NPs, have an extensive application in the development of new technologies in several areas, such as electronics, material sciences and medicine at the nanoscale (Magudapathy *et al.*, 2001) and recently their potential as antimicrobials has also been explored (Banu and Rathod, 2013). Silver sulphide (Ag_2S) is a direct narrow-bandgap semiconductor with good chemical stability and good optical limiting properties (Meng *et al.*, 2012; Wang *et al.*, 2008), presenting bandgap values in the range of 1-2 eV (Jadhav *et al.*, 2013). This low bandgap energy makes Ag_2S an effective semiconductor material for solar photocatalytic applications, as a result of its high capability of absorbing a broad radiation spectrum (Kumari *et al.*, 2014). Thus, silver sulphide appears to be a promising material for conversion of solar energy into electrical energy (Jadhav *et al.*, 2013).

Ag_2S NPs have singular properties, which enable these particles to have several applications, such as in solar cells, photo-detectors, light emitting diodes and switches, photoconductors, IR detectors, magnetic field sensors, optical filters, super-ionic conductors, solar-selective coatings and room temperature oxygen sensors (Lu *et al.*, 2005; Wang *et al.*, 2008; Pourahmad, 2012; Anthony, 2009). Other new applications have been presented, like NIR emitters for *in vivo* imaging (Zhang *et al.*, 2012; Hong *et al.*, 2012) and substrates for surface enhanced Raman scattering (SERS) spectroscopy (Hou *et al.*, 2012).

In spite of the wide range of possible applications for Ag_2S, little research has reported alternative methods for the synthesis of Ag_2S. The synthesis of this metal sulphide has been studied by hydrothermal, sol–gel and solvothermal methods (Yang *et al.*, 2013; Subash *et al.*, 2012; Mo *et al.*, 2012). Cubic Ag_2S was synthesized via hydrothermal method by using CTAB as a surfactant (Dong *et al.*, 2008) and other stabilizing agents, such as hexadecylamine (HDA), octylamine (OA), ethylenediamine (EDA) and dioctylamine (DOA) were used to synthesize stable Ag_2S NPs (Lim *et al.*, 2004). Nanocrystalline Ag_2S thin films were prepared by Chemical Bath Deposition technique and Successive Ionic

Layer Adsorption and Reaction (SILAR) technique at room temperature, using a silver nitrate– ammonia–thiourea system (Jadhav *et al.*, 2011). Recently, Kumari and colleagues (2014) focused their studies in the synthesis of renewable, eco-friendly, catalytic active and antimicrobial Ag_2S NPs for possible industrial application and bioremediation, in which the preparation of Ag_2S NPs was performed by one-step chemical co-precipitation method.

The use of living organisms such as bacteria, fungi or plants, for the production of metal NPs has also been studied. The biological methods present a huge potential to yield metal NPs with good control over their size distribution and are an eco-friendly and exciting alternative approach (Swathy, 2014). Some bacteria are commonly used in the preparation of nanoparticles of metals, oxides, and metal sulphides. For example, the bacteria of the genus *Shewanella* can reduce substances such as metal oxides, nitrates and sulphates, thus these bacteria have been used in the synthesis of gold nanoparticles (Suresh *et al.*, 2011a), arsenic sulphide nanotubes (Jiang *et al.*, 2009) and uranium dioxide nanoparticles (Burgos *et al.*, 2008). Klaus and colleagues (1999) have reported the biosynthesis of monoclinic *acanthite* (Ag_2S) NPs, with triangle and hexagon shapes, using *Pseudomonas stutzeri*. The synthesis of silver sulphide (Ag_2S) in a solution of $AgNO_3$ and sodium thiosulfate with *Shewanella* was also described (Suresh *et al.*, 2011b). The bacterial synthesis of silver sulphide NPs by the metal-reducing electrogenic bacterium *S. oneidensis* MR-1 was mentioned as a biocatalytic process demanding the presence of living cells in the reaction mixture of $AgNO_3$ and $Na_2S_2O_3$ salts (Debabov *et al.*, 2013).

Lately, an effluent containing sulphide, resulting from a bioremediation process treating real Acid Mine Drainage (AMD) has been used for the synthesis of metal sulphides and their nanocomposites, in particular, ZnS and ZnS/TiO_2 nanocomposite (Vitor *et al.*, 2015).

TiO_2 has photocalatytic properties, is widely available and is a nono-toxic, cheap and chemically stable material (Gude *et al.*, 2008). However, due its wide bandgap, around 3.2 eV (Yawalkar *et al.*, 2001), TiO_2 absorbs mainly in the UV region (Robert and Malato, 2002) and therefore, it has been mentioned as inappropriate material form direct use on environmental processes (Franco *et al.*, 2009). The use of metals and non-metal dopants (Ji *et al.*, 2010; Zhang *et al.*, 2008), co-catalyst loading (Arifin *et al.*, 2013; Mizukoshi *et al.*, 2010) and combinations with semiconductors, such as metal sulphides (Baker and Kamat, 2009; Balis *et al.*, 2013; ThanhThuy *et al.*, 2012) have been studied to overcome these disadvantages. TiO_2 has been used as substrate, thus, using sulphate reducing bacteria, composites of TiO_2 particles with biosynthesized ZnS nano-phases were manufactured via deposition of the metal sulphide at the substrate surfaces (da Costa *et al.*, 2012).

The process described by Vitor and colleagues (2015) consists in a bioreactor inoculated with a Sulphate-Reducing Bacteria (SRB) community, previously enriched from wastewater treatment plant sludge, to treat real AMD with pH values ranging between 2.0 and 2.5 and containing high sulphate ion concentrations (> 2g L^{-1}) and some metals, such as iron, copper and zinc. The bioremediation process relies on the activity of SRB that allows a consistent reduction of the sulphate present in the AMD to sulphide, which then precipitates the metals dissolved in the AMD as metal sulphides, decreasing both sulphate and metal's concentration in the resulting effluent to levels below the Maximum Recommended Values (MRV) for irrigation waters, according to Portuguese legislation (Decreto-Lei nº 236/98, 1998) (Vitor *et al.*, 2015). However, the produced effluent contains an excess of biologically generated sulphide, which can then be used for additional synthesis of metal sulphides and respective nanocomposites.

In the present research, Ag_2S NPs were synthesized both in batch and coupled to the continuous AMD bioremediation system, using the bioremediation system's effluent, containing surplus biogenic sulphide. Besides the economic advantages resulting from the synthesis of functional NPs, the process allows a marked decrease in the (toxic) sulphide concentration, which would otherwise present a serious environmental hazard.

2. MATERIALS AND METHODS

2.1. Synthesis of Ag_2S in batch

The effluent used for the batch synthesis tests contains sulphide and came from the AMD bioremediation system described above, which consists of a bioreactor with a SRB community and was already described in detail by Vitor and colleagues (2015).

The bioreactor's effluent was collected, filtered with filter 0.22 µm (syringe filter, Puradisc FP 30, Whatman) and placed in 120 mL glass bottles. The bottles were capped with butyl rubber stoppers and a needle was placed in the rubber to relieve the pressure. 10 mL of the silver (I) standard solution of 1000 mg L^{-1} of

Ag in 5% of HNO_3 (Alfa Aesar, Specpure) was added drop-by-drop to each batch, in order to obtain a final Ag(I) concentration of 100 mg L^{-1}. This procedure was performed under magnetic stirring. The batch assays were incubated at room temperature and carried out in duplicate, with all material sterilized by autoclaving.

The analysed parameters of the filtrated bioreactor's effluent: pH, redox potential (Eh), sulphate and sulphide concentrations, are presented in Table 8-1. After the bioreactor's effluent addition to the silver (I) solution, samples were collected at different incubation times: after 1 hour and after 1, 3 and 7 contact days. In each sample the pH, Eh, sulphate- and sulphide-ion, and silver concentrations were measured.

Table 8-1 – Parameters of the filtrated bioreactor's effluent before addition to the silver (I) solution

Parameters	pH	Eh (mV)	Sulphate concentration (mg/L)	Sulphide concentration (mg/L)
Bioreactor's effluent	8.09	-395	44	156

The sulphide was in excess in comparison to silver (I) and the molar ratio (silver/sulphide) was about 1:5 in order to guarantee that the sulphide was enough to precipitate all the silver (I).

A control assay was performed in the experimental conditions described, but using a sodium sulphide (Na_2SO_3, Panreac) solution (120 mg L^{-1} sulphide) in place of the bioreactor's effluent.

2.2. Ag_2S and Ag_2S/TiO_2 synthesis: continuous tests

The biosynthesis system consisted of a reaction vessel (1L, ISO flask), containing a known volume of silver (I) solution of known concentration under magnetic stirring where the bioreactor's effluent from the AMD bioremediation process and containing biogenic sulphide-ion is added drop-by-drop at a flowrate of 45.3 mL/day, at room temperature and pressure. The silver (I) solution was prepared by dissolving anidrous silver sulphate (Ag_2SO_4, 99.8%, VWR Prolabo) in ultra-pure water (milli-Q), yielding concentrations of around 3.06 mM (the real concentration of silver (I) in the solution was determined afterwards, by flame

AAS). The metal solution was deaerated in an ultrasound bath (J.P. Selecta Ultrasons- H) for 15 min, immediately prior to synthesis start.

The AMD used to feed the bioremediation system during the time period in which every synthesis took place was collected on July 2014, at the stream of S. Domingos (N: 37°39'38" W: 7°30'18"), located in the village of S. Domingos, Mértola, Southeast Portugal, and whose composition is presented in Table 8-2.

Table 8-2 - Typical chemical parameters and metals and sulphate concentrations in AMD and in the bioreactor's effluent used in the present work

	E_h (mV)	pH	$[SO_4^{2-}]$ (mg L^{-1})	$[S^{2-}]$ (mg L^{-1})	[Fe] (mg L^{-1})	[Cu] (mg L^{-1})	[Zn] (mg L^{-1})	[Al] (mg L^{-1})
AMD	398	2.58	2323	-	177.875	21.147	37.942	211
Effluent (typical)	-277	6.08	284	383.26	0.43	0.09	< 0.5	-

The bioreactor's effluent may contain bacteria and other metal sulphide particles dragged from the bioreactor (BR) by the effluent. Therefore, in order to prevent possible contamination of the synthesized particles, a two-stage filtering system was set in line with the system, between the bioreactor and the reaction vessel, consisting of a small glass column (~14 cm long, 2 cm Ø) with glass wool pre-filter, for retaining the larger particles, followed by a 0.2 μm pore size syringe filter (Whatman Puradisc 25 AS). Care was taken to minimize sulphide losses along the effluent line, and thus the length of the tube between the effluent and the reaction vessel was minimized.

The volume of sulphide-rich effluent added to the silver (I) solution was calculated in each case to yield a 1:1.2 molar ratio (silver/sulphide), based on the sulphide concentration measured at the bioreactor outlet, the metal solution concentration and the reaction stoichiometry. This ratio was decided upon (contrary to the ratio of 1:5 used in previous batch studies) according to the results obtained for other metallic sulphide synthesis (data to be published), since it was found that, while the addition of excess of sulphide increases the metal precipitation, evidence of solid sulphur was also found on some precipitates, probably due to a too large excess of sulphur. Hence, it was considered preferable to reduce the sulphide excess added, in order to try to obtain higher purity particles.

The synthesis of the Ag_2S/TiO_2 composites was performed by adding 0.06 mg of commercial titanium (II) oxide (TiO_2) powder (Evonik Industries Aeroxide P25) to the metal solution, per 50 mL of metal solution (da Costa *et al.*, 2013), prior to deaeration. The particle size of the TiO_2 used was 21 nm (according to the manufacturer information, Evonik Industries, 2007).

The synthesized particles were then separated by centrifugation twice at 4000 rpm, for 15 min (Rotofix 32A, Hettich), washed 3 times with 70% ethanol and finally dried under vacuum (desiccator connected to a vacuum pump), saving a sample of each synthesis supernatant for further analysis.

2.3. Analytical methods

Samples from batch synthesis tests were periodically collected, using sterile syringes, for analysis, while in the continuous experiments, after the end of the synthesis, the remaining supernatant was analysed. Sulphide concentration was measured immediately after sampling, using a UV–Visible spectrophotometer (DR 2800, Hach-Lange) by the Methylene Blue Method (665 nm, Hach-Lange). The samples were then centrifuged at 4000 rpm for 5 min, for subsequent analysis of redox potential, pH, sulphate-and silver-ion concentration. Redox potential and pH were determined using a pH/E Meter (GLP 21, Crison) and sulphate concentration was quantified by UV-visible spectrophotometry (Hach-Lange DR2800 spectrometer) using the SulfaVer®4 method. The silver concentration was determined by flame atomic absorption spectroscopy (Flame-AAS) using an Analytikjena NovAA 350 model spectrometer.

2.4. Precipitates characterization

The precipitates obtained were recovered by centrifugation at 4000 rpm for 10 min and the pellets were washed with ethanol 70% (stirred and centrifuged 4000 rpm for 20min, 3 times) and the precipitates were then dried in vacuum (Binder, VDL) at 37°C ± 1°C. The dried precipitates were then characterized by X-Ray Diffraction (XRD), by Scanning Electron Microscopy (SEM) coupled to an Energy Dispersive X-ray Spectrometer (EDS) and Transmission Electron Microscopy also coupled to an Energy Dispersive X-ray Spectrometer (TEM-EDS).

The XRD analysis was performed using a PANalytical X'Pert Pro powder diffractometer, operating at 45 kV and 30 mA, with Cu Kα radiation filtered by Ni. The XRD patterns were recorded using an X'Celerator detector, with a step size (2θ) of 0.001°, and a time per step of 19.7 s. Peak analysis and crystalline phase identification were conducted using the High-Score Plus software, with the ICDD PDF-2 database. For the precipitates from continuous experiments, the diameter of the synthesised particles (Dp) was also estimated using the Scherrer equation (Scherrer, 1922), based on the form factor (K, 0.94) and considering the most intense peak observed for each crystalline structure present.

For the determination of the particles morphology and size, from batch studies, a SEM (Hitachi, 3700N) and TEM (Hitachi, H8100 model, with a LaB6 filament) analysis were made coupled to an EDS (Bruker, Xflash 5010 and ThermoNoran, respectively) for the determination of the precipitate's elemental composition. However, the morphology and size of the particles from the continuous experiments were determined in other equipment or in other conditions, thus, a SEM coupled with Energy-Dispersive X-ray Spectroscopy (EDS or EDX) analysis was carried out in a SEM-FIB (Zeiss Auriga) instrument, at an accelerating voltage between 0.1 to 30 kV. The samples were prepared by deposition of the precipitate directly onto the carbon tape, which was then coated by carbon evaporation. The elemental analysis was carried out on a semi-quantitative EDS (Oxford XMax 150). TEM coupled to EDS analysis was performed also in a Hitachi H-8100 electron microscope, operating at 160kV. Droplets containing the obtained precipitates were prepared and placed on a copper grid and dried at room temperature.

Moreover, the particles from the continuous synthesis were also characterized by Diffuse Reflectance Spectroscopy (DRS) and the Zeta potential was measured.

In DRS, the UV-Vis Spectrometer UV-2600, Shimadzu was used to determine the percentage of diffuse reflectance value (%DR) of each sample. The values of the %DR were measured between 200 and 1400 nm. The samples were prepared using a sample holder containing barium sulphate ($BaSO_4$, Sigma-Aldrich) as base. The samples were spread in this base and uniformed for subsequent introduction of the sampler in the integrating sphere spectrometer.

The Zeta potential measurements were performed in a Nano ZS Malvern equipment, using Capillary cell Folded cells. Aqueous solutions at pH values between 2 and 8 were previously prepared using solutions of 5 M NaOH or HNO_3 65% to adjust pH. The particles were added to the different solutions and subjected to ultrasound for 1min, prior to analysis.

3. RESULTS AND DISCUSSION

3.1. Batch synthesis experiments

A batch synthesis experiment was performed using the bioreactor's effluent with an excess of biogenic sulphide, as described. The sulphide has its own environmental hazards (Mandal *et al.*, 2006; Labrenz and Banfield, 2004), thus the used of the excess of sulphide present in this effluent for silver (I) removal and recovery can have a double advantage: treat an effluent with excess sulphide and simultaneously recover silver (I) from an aqueous solution.

The chemical parameters of the bioreactor's effluent used in the batch synthesis tests are shown above in Table 8-1 and the silver solution had a concentration of 100 mg L^{-1} of Ag(I).

The addition of the silver (I) solution to the bioreactor's effluent formed a dark precipitate instantaneously and was followed by all silver (I) removal from the solution (100%) and by a decrease in sulphide concentration. These results showed that silver (I) can be removed from an aqueous solution instantaneously using a bioreactor's effluent with excess sulphide, as shown in Figure 8-1.

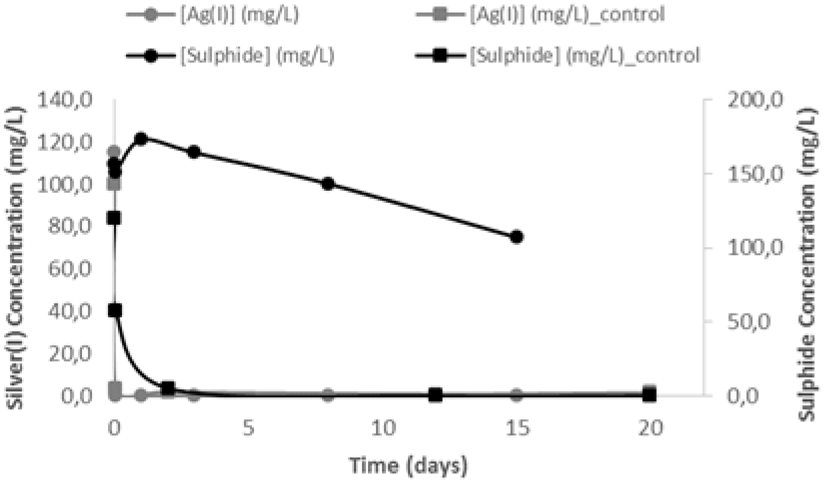

Figure 8-1 – Silver (I) (grey symbols) and Sulphide (black symbols) concentration in the both batch experiments, using bioreactor's effluent (Circles) and using a chemical sulphide solution (Squares) for silver (I) precipitation. The sulphide solution serves as a chemical control. Data are the average of duplicates and the error bars indicate the standard deviations of the average values (in all cases error bars are smaller than symbols, therefore not visible).

The interaction between sulphide and silver ions occurred within the first minutes, and the decrease of sulphide concentration during the rest of the assay can be related to the H_2S volatility, since whenever a sample was collected, there was some sulphide loss. Despite the fact that the decrease of sulphide concentration in the batch experiments using the bioreactor's effluent was not as high as in the experiment where a chemical sulphide solution (control) was used, this decrease was sufficient to remove all the silver from the solution, possibly in the form of Ag_2S. In both experiments a total removal of silver was achieved almost immediately.

The pH decreased during the synthesis, from values of 8 to 2; this fact can be justified by the initial low pH value in the silver (I) solution (<1), which contributes to the pH decreased in the mixture. This fact can also be related with the dissociation of the hydrogen from the H_2S as the sulphide reacts with silver (I) ions, producing Ag_2S.

The redox potential increased from -395 to 34 mV and from 0 to 280 mV in experiment using bioreactor's effluent and in the control, respectively. These results can be justified by the removal of the metallic species from the solution.

In order to determine the chemical and conductor nature of the precipitates and to evaluate the morphology and size of their particles, a complete characterization of both precipitates obtained in batch and in continuous were performed. This information is highly relevant, considering the possible utilization of these particles as catalyst and /or as components of low cost solar cells.

3.1.1. Precipitate characterization

The precipitate obtained in the batch experiments was washed and dried for subsequent characterization by XRD, SEM-EDS and TEM-EDS. These analyses were performed to determine the crystallography of the synthesized particles and the particles morphology and size, respectively.

The XRD diffractograms of silver precipitates are shown in Figure 8-2.

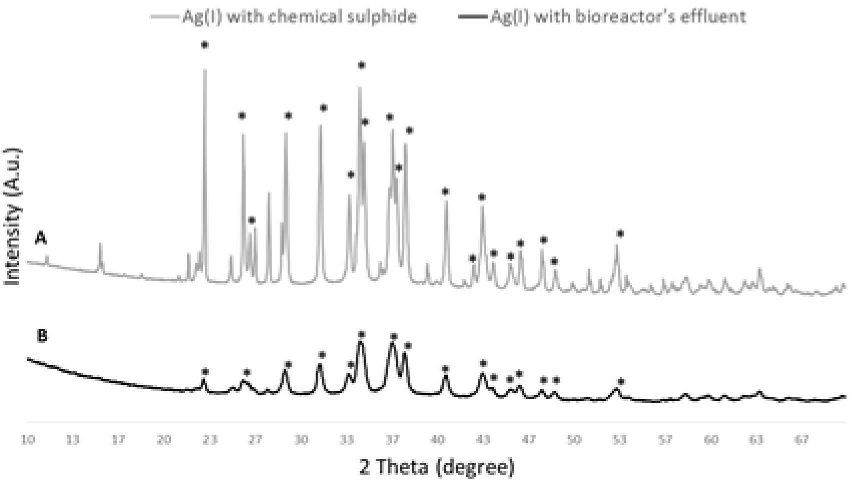

Figure 8-2 - X-Ray powder diffraction patterns for precipitates generated in batch assays using a chemical solution of sulphide (**A**) and bioreactor's effluent from an AMD bioremediation process (**B**) for silver (I) removal. (Ag_2S - *acanthite* in a monoclinic crystalline phase ('*')).

The XRD analysis for the materials synthesized both with chemical sulphide solution (Figure 8-2 A) and with the bioreactor's effluent (Figure 8-2 B), may be matched with *acanthite* (Ag_2S) in a monoclinic crystalline phase, since the correspondent identified patterns, represented in Figure 8-2 A and B with an '*', are consistent with *acanthite* (ICDD PDF2 # 00-024-0715). However, the peaks are more defined for the precipitate obtained with chemical sulphide (A). This fact is probably related to the smaller size of the particles obtained in the assay using the bioreactor's effluent, which results in more elongated peaks in the XRD diffractogram.

In order to confirm the XRD results, SEM-EDS and TEM-EDS analyses were performed for the precipitates obtained using the bioreactor's effluent.

Figure 8-3 presents the SEM and EDS results for the samples obtained using the bioreactor's effluent.

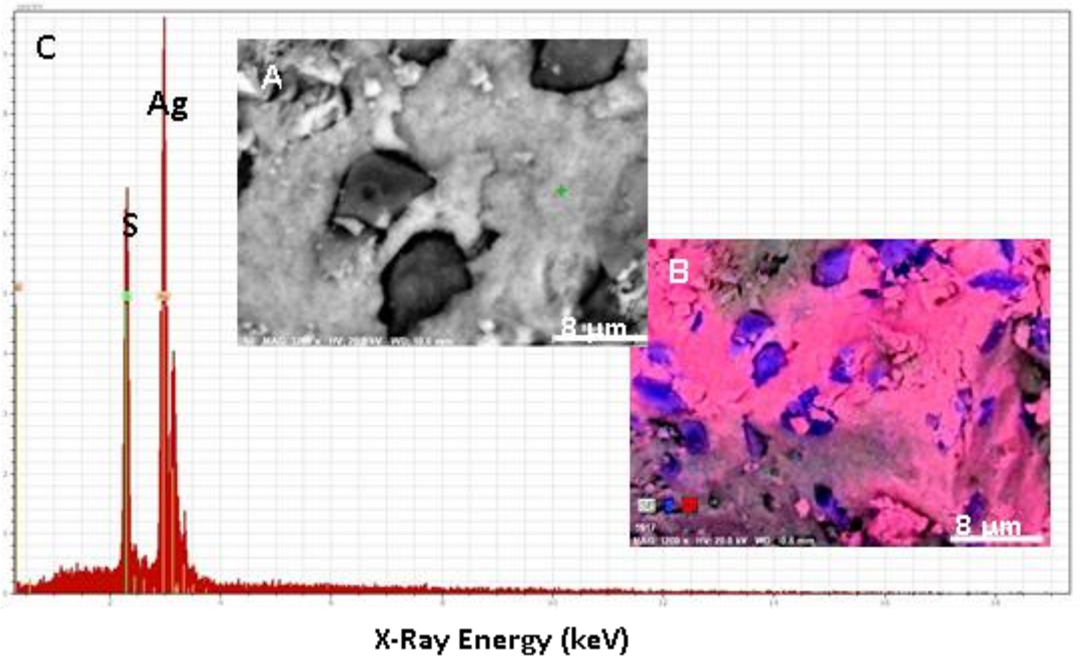

Figure 8-3 - SEM image (A), elements XEDS mapping (B) and corresponding EDS spectra (C) for the particles obtained using the bioreactor's effluent with an excess of sulphide for silver (I) precipitation from an aqueous solution.

The SEM image (Figure 8-3 A) is inconclusive about the morphology of the particles; however the XEDS mapping and the corresponding EDS spectra confirmed the results obtained by XRD, since the elements mapping (Figure 8-3 B) demonstrated that silver, in red, and sulphur, in blue, are overlapped, showing for that reason a pink color. This analysis was followed by EDS, which reveal that this particle is composed by Ag and S in an atomic percentage of 53% and 47%, respectively. Considering that the precipitate is Ag_2S, as showed by XRD, the ratio between Ag and S should be 2:1, however in this sample the ratio is 1.1:1.0. This result can be justified by the presence of sulphur in the precipitate, which can be due to the excess of sulphide used in comparison to silver (I). In fact, at the end of the synthesis the supernatant still had around 100 mg L^{-1} sulphide ion concentration. The sulphur in excess can also be seen in the XEDS mapping, represented in blue and not combined with silver.

As mentioned above, it was not possible to determine the particle morphology by SEM analysis. As such, a TEM coupled to EDS analysis was performed, as an attempted to determine the particles size and morphology. The results obtained are presented in Figure 8-4.

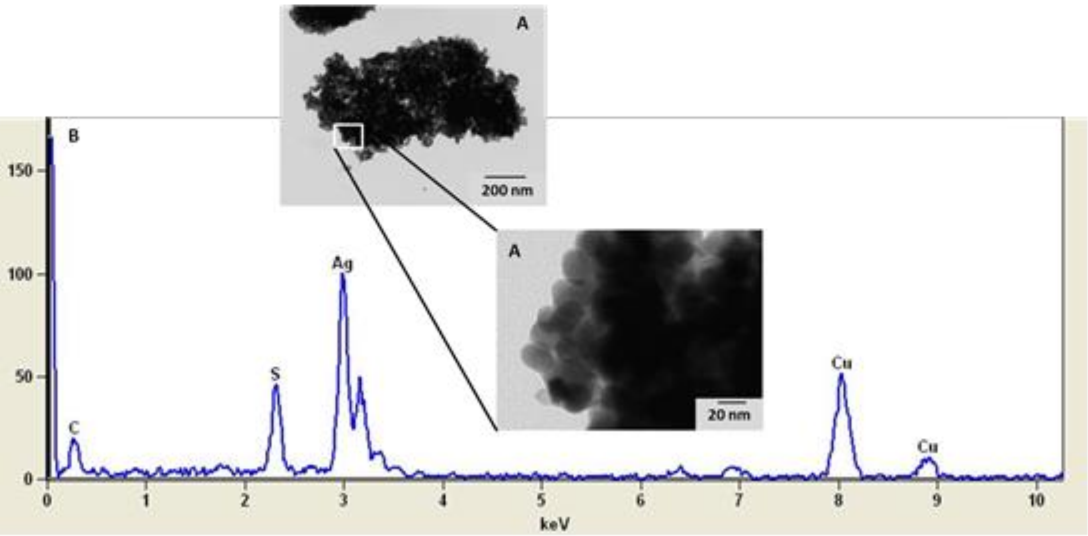

Figure 8-4 - TEM images for the nanoparticles of Ag_2S (A) and with the corresponding EDS (B) of the precipitate obtained in batch assays using the bioreactor's effluent from an AMD bioremediation process.

The TEM analysis allowed to conclude that the Ag_2S particles, present a spherical and spheroidal morphology with sizes ranging between 18-29 nm and 28-54 nm (Figure 8-4 A), respectively. This fact confirms the hypothesis suggested by the XRD analysis that the Ag_2S particles obtained in the batch experiment have very small sizes, thus responsible for elongated peaks in the XRD spectra. Cu element identified in the EDS spectrum belongs to the sample grid.

3.2. Ag_2S and Ag_2S/TiO_2 synthesis: continuous tests

3.2.1. Bioremediation process: Characteristics and performance

A scale-up of the batch system for a continuous system was made, in order to understand if it would be possible to obtain similar results in a continuous system, using the effluent with excess sulphide produced in the AMD bioremediation system.

3.2.2. Biosynthesis of Ag_2S and Ag_2S/TiO_2

Thus, the synthesis of Ag_2S downstream to the bioreactor and using the optimized conditions tested in batch, that allowed the removal of all the silver (I) present in aqueous solution was performed.

The percentage of silver (I) removal in the continuous system was about the same for either the "simple" synthesis (only Ag_2S) and for the TiO_2 composite (i.e., 100%).

Ag_2S NPs synthesis was already reported by Debabov and colleagues (2013), however the maximum synthesis yield obtained was 53% and this result is far from the result obtained in the present work (100%).

3.2.3. Precipitate's characterization and potential applications

3.2.3.1. X-Ray diffraction (XRD)

The XRD patterns of the precipitates and respective TiO_2 composites obtained in the continuous experiments are presented in
Figure 8-5.

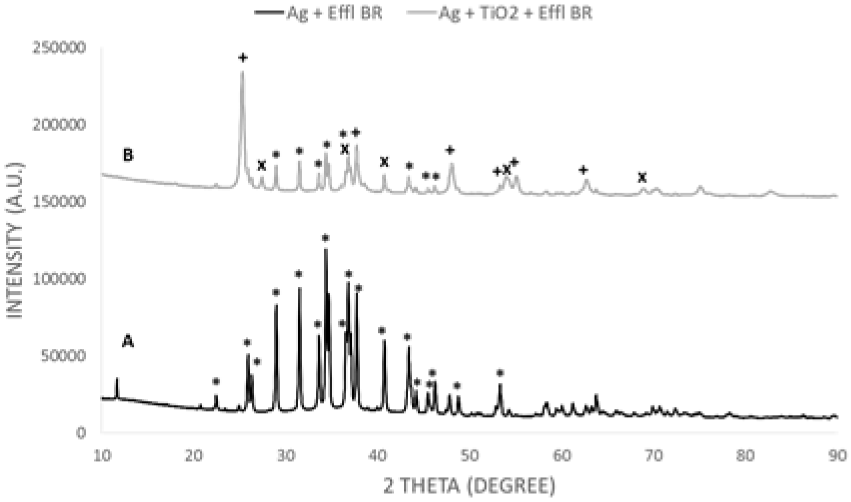

Figure 8-5 - X-ray powder diffraction patterns for the precipitates obtained in the system coupled to the bioremediation process in the absence (A) and presence of TiO_2 (B) using the effluent from the bioreactor and also peaks correspondent to silver (I) sulphide. (Ag_2S - acanthite in a monoclinic crystalline phase ('*'); Ag_2S/TiO_2 - *anatase-* ('+') and *rutile*-structured TiO_2 ('x')).

In the XRD diffractogram presented in
Figure 8-5 A, the predominant crystalline phase present has been identified as monoclinic Ag_2S (*acanthite*), which is in accordance to the previous batch studies (Figure 8-2). Particle size estimated using the Scherrer equation (Sherrer, 1922), was around 45 nm for the Ag_2S crystallites.
The commercial TiO_2 used had already been characterized. The data supplied by the manufacturer and previous studies (da Costa *et al.*, 2012; Vitor *et al.*, 2015) reported that TiO_2 presented peaks characteristic of both *anatase* and *rutile* structures, indicating the presence of a mixture of both crystal phases, with the average TiO_2 crystallite size estimated at about 21 nm. In the XRD diffractogram of the composite Ag_2S/TiO_2 synthesized (Figure 8-5 B), it is

possible to identify characteristic peaks for *anatase*- ('+') and *rutile*-structured TiO_2 ('x') and also peaks correspondent to silver (I) sulphide, acanthite in a monoclinic crystalline phase ('*'), although some peaks are overlapped. The estimated size for silver sulphide particles in the composite, using Scherrer equation, was at about 44 nm.

3.2.3.2. SEM-EDS

Figure 8-6 present SEM images of the Ag_2S particles and Ag_2S/TiO_2 composites synthesized in the continuous system.

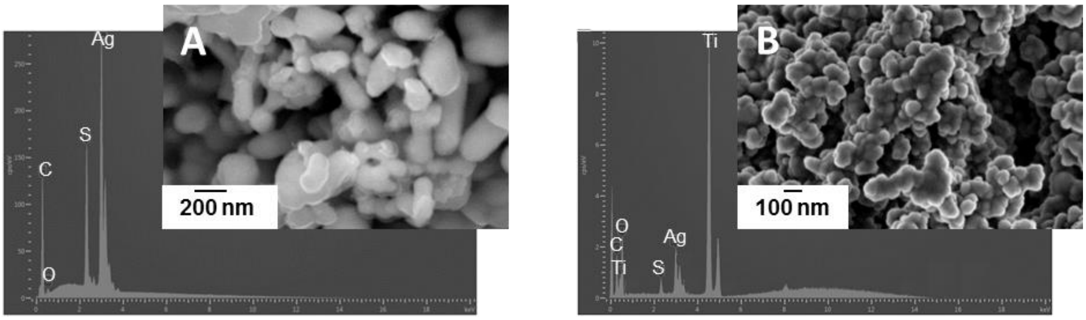

Figure 8-6 - SEM images for Ag_2S and Ag_2S/TiO_2 composite particles synthesized coupled to the AMD bioremendiation continuous process (**A** and **B**, respectively) with the correspondent EDS.

The EDS analysis, Figure 8-6 A and 6 B, confirmed that both particles contain the elements Ag and S, which is consistent with the formation of Ag_2S, as suggested by XRD and as predicted by batch results. In the composites and as expected, beyond the Ag and S, Ti was also identified by EDS.

3.2.3.3. TEM-EDS

TEM micrographs of the particles and composites of Ag_2S synthesized coupled to the AMD bioremendiation continuous process are shown in Figure 8-7.

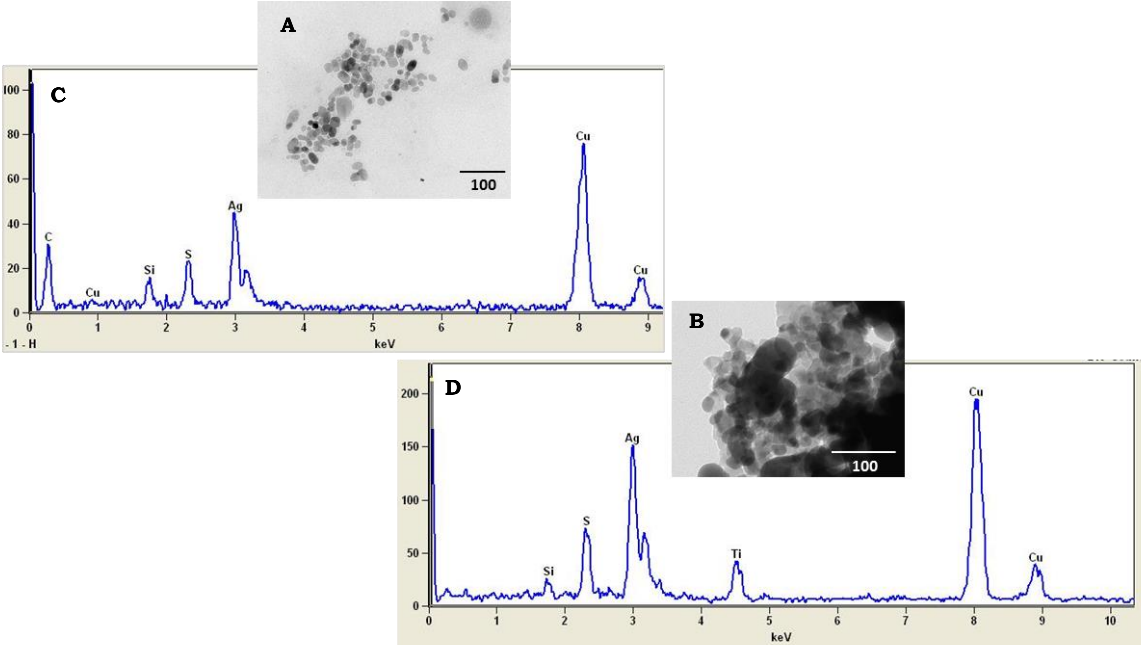

Figure 8-7 - TEM micrographs for Ag_2S precipitate and Ag_2S/TiO_2 composite obtained in the system coupled to the AMD bioremediation continuous process (**A** and **B**, respectively) and correspondent EDS (**C** and **D** for Ag_2S and Ag_2S/TiO_2 particles, respectively).

The TEM image of Ag_2S precipitates (Figure 8-7 **A**), produced coupled downstream the AMD bioremediation process, showed that the particles feature a spheroidal and cylindrical morphology with particles size ranging between 15 nm and 24 nm. Other authors have reported Ag_2S nanocrystals with an average size of 25.7 nm in the absence of substract (Hou *et al.*, 2012). These results are closed to those obtained for the Ag_2S particles (15-24 nm) synthesized coupled to the continuous system, using biogenic sulphide present in the effluent.
TEM analysis of the Ag_2S/TiO_2 composite (Figure 8-7 **B**) revealed that the particles have a spheroidal morphology and a higher size, 30-60 nm, which is within the envisaged by Scherrer equation (44nm). This dimensions of the nanocomposite are probably associated to the deposition of Ag_2S in the surface of TiO_2. Similar results were already reported for other metal sulphide nanocomposite (ZnS/TiO_2), that mentioned that the surface of the TiO_2 particles are “decorated” with irregular deposition of metal sulphide nanoparticles (da Costa *et al.*, 2012), as seems to happen in the Ag_2S/TiO_2 nanocomposite.
The EDS analysis (Figure 8-7 **C** and **D**) confirmed the results obtained by XRD and SEM-EDS for both precipitates.

3.2.3.4. Diffuse Reflectance Spectroscopy (DRS)

The optical characterization of the Ag_2S based samples was carried out by measuring their diffuse reflectance, R, at room temperature. R can be related to the absorption Kubelka-Munk function F_{KM}, by the relation $F_{KM}(R) = (1-R)^2/2R$, which is proportional to the absorption coefficient (Kortuem, 1969). The obtained absorption spectra are shown in Figure 8-8.

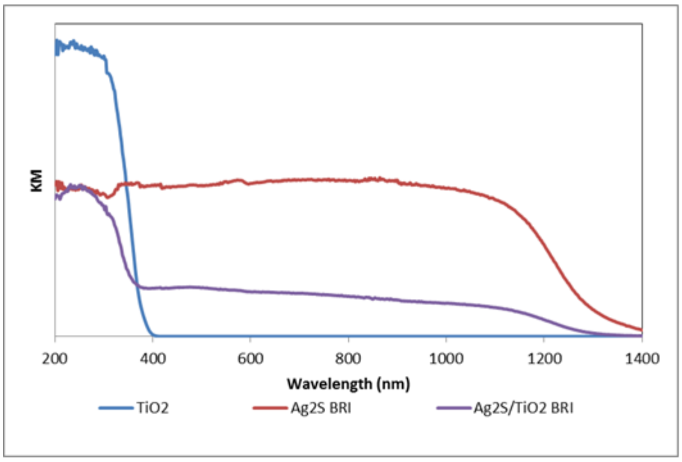

Figure 8-8 - Absorption spectra of nanoparticles and nanocomposites of silver sulphide obtained and commercial TiO_2.

Analyzing Figure 8-8, it is noted that Ag_2S nanoparticles absorb in all the visible range of the electromagnetic spectrum. The optical behavior of the Ag_2S/TiO_2 composite indicates that these particles are able to absorb in the visible range, due to the presence of Ag_2S.

Blue shifts in the optical absorption band edges of the Ag_2S and TiO_2 components can be seen for the Ag_2S/TiO_2 sample compared to the TiO_2 powder and Ag_2S nanoparticles. The optical bandgap energies of the samples, Eg, were estimated by plotting the function $f_{KM} = (F_{KM} h\nu)^2$ vs. $h\nu$ (Tauc plot), where h stands for Planck's constant and ν for the radiation frequency, and by extrapolating the linear portion of the curve to zero absorption (not shown). The extrapolated Eg values are given in Table 8-3.

Table 8-3 – Bandgap energy values determined for the silver sulphide nanoparticles and nanocomposites synthesized

	TiO_2	**Ag_2S BR**	**Ag_2S/TiO_2 BR**
Bandgap (eV)	3.49	0.98	3.48; 1.01

The bandgap energy for the TiO_2 nanoparticles was 3.49 eV and, as expected, not significant changes were observed after Ag_2S modification. The direct band gap of the silver sulphide (Eg ~1 eV) makes this semiconductor be used as a solid-state electrolyte, presenting both ionic and electronic conduction at room temperature (Hussain *et al.*, 2012; Wang and Qi, 2008). This Ag_2S bandgap and the large absorption coefficient turns silver (I) sulphide an effective semiconductor material for photovoltaic application (Meng *et al.*, 2012).

The Ag_2S nanoparticles prepared present a lower band gap energy compared with TiO_2, allowing the use of visible radiation by the Ag_2S/TiO_2 nanocomposite. This semiconductor nanocomposite presents two absorption edges related with the presence of Ag_2S and TiO_2 particles. It is also interesting to note that the bandgap energy of the Ag_2S nanoparticles prepared without the presence of TiO_2 is red shifted in comparison with Ag_2S/TiO_2. This result suggests that the presence of TiO_2 has some influence on the Ag_2S particle size, being smaller the particles prepared without it.

3.2.3.5. Zeta Potential

The Zeta (ζ) potential analysis allows the determination of the surface charge of nanoparticles suspended in solutions. The nanoparticle's surface charge attracts a thin layer of ions of opposite charge, which move with the nanoparticle as it diffuses throughout the bulk solution. The ζ-potential is the electric potential at the boundary of this double layer, and typical values range between -100 mV and +100 mV, depending on the characteristics of the solution. The colloidal stability depends on the particle's ζ-potential magnitude. Thus, nanoparticles with ζ-potential values ranging between -30 mV and +30 mV in a suspension tend to aggregate, a fact related to Van der Waals inter-particle attractions. The nanoparticles with ζ-potential values higher than +30 mV or lower than -30 mV usually display a relatively high stability (Mandzy *et al.*, 2005).

Figure 8-9 presens the ζ-potential values measured for the silver sulphide nanoparticles and nanocomposites synthesized.

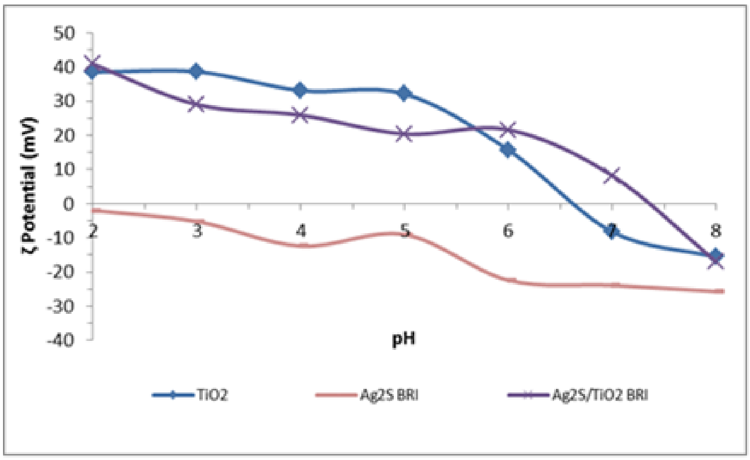

Figure 8-9 - ζ-potential values of the nanoparticles and nanocomposites of silver sulphide obtained in continuous system.

Taking into account the results presented in Figure 8-9, it is possible to conclude that all Ag_2S nanoparticles synthesized are able to suffer aggregation in aqueous media due to Van der Waals forces, since their zeta potential values are within the range of +30 mV to -30 mV. The commercial TiO_2 is stable between pH 2 and pH 5.5, from which the values of zeta potential decreases considerably, crossing the x-axis's to pH 6.5 (isoelectric point) and then keeping a negative charge in the particle's surface.

The Ag_2S nanoparticles synthesized in line with the bioremediation systems do not exhibit an isoelectric point, keeping the particle surface charge negative along all the pH range tested, indicating that these nanoparticles are best suited as catalysts for the degradation of cationic emerging pollutants, thereby promoting the affinity of the charges at the particles surface.

According to Debabov and colleagues (2013) some parameters, such as, reagent concentration, temperature and cell incubation time did not affect significantly the Ag_2S NPs size generated using *S. oneidensis*, however, longer incubation times improved the yield, being the maximum yield achieved of 53% (Debabov *et al.*, 2013). Comparing to the results obtained in the present study, the use of a bioreactor's effluent with an excess of sulphide allows achieving a higher yield (100%), also obtaining Ag_2S NPs. Thus, the use of the biologically generated sulphide present in an effluent is a clear advantage comparing to other biological synthesis processes used to produce silver sulphide NPs, as it removes the excess sulphide from the produced effluent (which would otherwise pose an environmental hazard), while simultaneously removing/recovering silver from an aqueous solution.

The biological synthesis of Ag_2S was already studied using, for example, *Pseudomonas stutzeri* (Klaus *et al.*, 1999) and *Shewanella oneidensis* (Suresh *et al.*, 2011a, 2011b). In the first case, the Ag-containing crystals were embedded in the organic matrix of the bacteria *P. stutzeri* and the crystals were identified as Acanthite crystals with a size up to 200 nm and the particles accumulated in the cells (Klaus *et al.*, 1999). The use of *Shewanella oneidensis*, allowed to obtain an efficient generation of extracellular silver sulphide NPs, capped by a detachable protein/peptide surface coat with a yield of 85% (Suresh *et al.*, 2011a, 2011b). However, in the present study a similar result was obtained, crystalline *acanthite* NPs, without the need of cells and with a yield of 100%.

These results confirm the relevance of the method described to produce materials with potential interest for photovoltaic application by using biogenenic sulphide as a precipitate's agent of silver (I) from aqueous solutions.

4. CONCLUSIONS

In the present work, the use of biogenic sulphide from an effluent generated in an AMD bioremediation process, has proven be an efficient process for the synthesis of Ag_2S nanoparticles and Ag_2S/TiO_2 nanocomposites. The synthesis of Ag_2S was carried out in a batch with high performance, producing NPs with spherical and spheroidal morphology and particle size ranging between 18-29 nm and 28-54 nm, respectively. Hence, the synthesis process was coupled downstream to the continuous AMD bioremediation system. The Ag_2S NPs obtained in this case feature a spheroidal and cylindrical morphology with particle size ranging between 15 nm and 24 nm. Ag_2S/TiO_2 nanocomposite was also produced coupled downstream to the continuous AMD bioremediation system, and the TEM analysis revealed that the particles have a spheroidal morphology and a higher size, 30-60 nm, probably due to the deposition of Ag_2S in the surface of TiO_2.

The synthesized Ag_2S NPs and respective TiO_2 nanocomposite have semiconductor properties that might confer several applications to those particles, for example, photovoltaic application. All silver (I) sulphide NPs and nanocomposites obtained using the bioreactor`s effluent exhibit a superficial negative charge, which will condition their possible applications.

Therefore, the synthesis processes described here are economic and eco-friendly alternatives, since they produce metallic sulphide nanoparticles with potential applications and, simultaneously, use an effluent, with biogenic sulphide, which would otherwise present an environmental hazzard.

Further work is currently underway to study other potential applications of the Ag_2S nanoparticles and respective TiO_2 nanocomposite, namely radiation based applications.

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CHAPTER 9

Conclusions and Future Perspectives

CONCLUDING REMARKS

The search for feasible approaches to recover precious metals from secondary sources, for instance, industrial effluents contaminated by those metals, is extremely important. Therefore, in the present work two methods, chemical (liquid-liquid extraction) and biological (using anaerobic bacteria) were investigated.

Hence, the present work provides a broad spectrum of results that highlight innovative bacterial-based strategies for the removal and recovery of precious metals as NPs. Although most of the investigation described in this work relates, mainly, the biorecovery of some precious metals using anaerobic bacteria communities, or products resulting from their activity, studies to determine the bacteria involved in such biorecovery processes were performed and a simple fingerprinting method to identify cloned 16S rRNA genes with similar sequences, avoiding the need to sequence redundant clones, was demonstrated to be effective.

Beyond these biological processes, part of this PhD was dedicated to the research of new extractants to be used in liquid-liquid extraction of platinum (IV) and palladium (II). In fact, one N,N'-tetrasubstituted malonamide derivative, the DMDCHTDMA, and two N,N'-tetrasubstituted succinamides, the DMDCHSA and DMDPHSA, showed to be promising extractants for the recovery of platinum (IV) from hydrochloric acid media, without the need of tin(II) chloride addition, as it has been deemed necessary in many studies similar to these. In addition, the platinum present in the loaded organic phases can be easily and quantitatively stripped to a new aqueous phase. In the Pt(IV) organic loaded phase with DMDCHTDMA the Pt(IV) was stripped by simple contact with 1 M HCl solution, while in loaded organic phases with the DMDCHSA and DMDPHSA a new stripping agent, never reported before, seawater, was efficiently used. All extraction processes were kinetically fast and the three extractants showed quite a good loading capacity towards platinum (IV). Another advantage demonstrated by the tested extractants was that they can be reutilized several times without losing their extraction ability. However, in some experiments 1,2-DCE was replaced by more environmentally friendly diluents, but Pt(IV) extraction was negatively affected. This fact could be a disadvantage in the application of this extractants in industrial processes. Furthermore, the liquid-liquid extraction, as most of the chemical processes used for precious metals removal, needs reagents that have a cost and that consequently produce more wastes and/or effluents. Hence, biological methods underlined on the use of naturally occurring organisms, can be a good alternative to overcome these disadvantages. For these reasons the subsequent studies were focused in the use of anaerobic bacterial consortia for precious metals recovery and finally on the possible combination between liquid-liquid extraction and biorecovery methods.

A bacterial community enriched from sludge from a wastewater treatment plant and composed mainly of *Clostridium* species was able to reduce Pd(II) to Pd(0) nanoparticles during bacterial growth in a minimal medium,

This clearly demonstrates the potentialities of using metal-resistant bacterial consortia simply enriched from waste samples for the bio-synthesis of metallic NPs. For the identification of the bacteria potentially involved in Pd(II) removal and simultaneously to understand the evolution of the bacterial consortium when subjected to different Pd(II) concentrations, a bacterial consortium was grown in the absence of Pd(II) and in the presence of 5 and 50mg/L of Pd(II). Thus in order to identify the main groups of bacteria that constitute the major part of the communities, a new simple and sensitive method that detect polymorphisms based on urea–agarose gel electrophoresis was tested and applied to select representative cloned amplicons for Sanger sequencing. Moreover, in order to study the feasibility of using only one of the primers, reducing the sequencing costs, the taxonomic classifications retrieved with sequences from one half side of the 16S rRNA gene was compared with classifications obtained with sequences from the other half side of the gene. The results showed that the percentage of bacteria belonging to genus *Clostridium sensu stricto* increased from undetected to 21 and 41%, respectively, for cultures without, with 5 and 50 mg L-1 Pd(II). This demonstrated that bacteria from genus *Clostridium sensu stricto* can be a potential key agent for the biorecovery of this metal. Thus, the use of a simple fingerprinting method to identify cloned 16S rRNA genes with similar sequences, avoiding the need to sequence redundant clones, was feasible and represented an important contribution to characterize bacterial communities. This research also contributes with knowledge about suitable partial 16S rRNA gene regions to be used in taxonomic studies.

The possibility of combining liquid-liquid extraction scheme with the use of bacterial communities for Pd(II) and Pt(IV) removal was tested. In those studies it was demonstrated that DMDCHSA can be efficiently used to extract Pd(II) to the organic phase and that seawater, which is an inexpensive and environmental friendly stripping agent, can be used as stripping agent to re-extract Pd(II) and Pt(IV) to a new and purified aqueous phase. The addition of a filtrated solution from bacterial growth to the palladium and platinum loaded stripping phases of seawater allowed to recover palladium and platinum sulphides as NPs or as particles with sizes lower than 150 nm, with potential functional applications.

The obtainment of metal sulphide NPs using a solution from the growth of anaerobic bacteria is directly related to the sulphide produced by sulphate-reducing bacteria present in the bacterial community. In our laboratory, we develop bioremediation processes to treat AMD using sulphate-reducing bacteria communities. The effluent resultant from these processes are treated water with an excess of biogenic sulphide. Thus, the ability of this effluent, containing biogenic sulphide, was tested for the recovery of gold (III) from aqueous media. This process allowed the recovery of gold (0) as metallic NPs. This substantiates the fact that this gold recovery is not only dependent on the sulphide ions available in the solution, but may be dependent on the presence of other compounds produced by the bacterial community in the bioreactor. The process described in this work can be a promising alternative for gold recovery from aqueous solutions. This NPs have a wide range of industrial applications, high market prices and extensive use. Furthermore, the use and valorisation of an effluent and the fact that the process is performed at room pressure and temperature demonstrates the environmental and economic advantages of this process.

The effluent, with biogenic sulphide, from an effluent generated in an AMD bioremediation process was also used for the synthesis of Ag_2S NPs and Ag_2S/TiO_2 nanocomposites. The Ag_2S NPs synthesis was first tested in a batch system and a yield of 100% was achieved. Therefore, a scale-up for a continuous system fitted downstream to the AMD bioremediation process was performed and small Ag_2S NPs, with sizes between 15-24 nm, were obtained. In addition, the successful synthesized of the respective TiO_2 nanocomposites was also achieved. The semiconductor properties, of these NPs, makes them potentially functional for applications based on radiation uses.

In summary, the results obtained in this PhD thesis are important findings emphasizing, besides the evaluation of new strategies for precious metals removal and/or recovery, the importance of finding efficient, low cost and "green" technologies, for instance aiming the valorisation of an effluent containing biogenic sulphide.

FUTURE PERSPECTIVES

The recovery of precious metals is interesting due to their high market prices along with various industrial applications. These applications gain more importance if the obtained metal particles are NPs. However, the availability of precious metals is very limited, thus the recovery of these metals from aqueous and waste solutions by biologic activity can be an economically attractive approach. In the present work it was demonstrated the effectiveness of an effluent, with biogenic sulphide, from an AMD bioremediation process, for gold and silver recovery from aqueous solutions, as NPs. Therefore, I intend to explore in future works the potential of this effluent in the removal/recovery of other metals, such as, palladium and platinum. The effluent from an AMD bioremediation process should also be tested using real effluents contaminated by precious metals for their recovery. The process conditions should also be studied and optimized for that purpose.

Some applications of the NPs obtained were already known. Thus, the NPs applications, namely in chemical industry, petroleum refining and automotive catalysts and in electronics industry, must be tested, not only for the applications already known, but also for new applications, such as in photocatalytic processes and for example in the treatment of pharmaceutical and hospitalar effluents.

Appendixes

APPENDIX 1

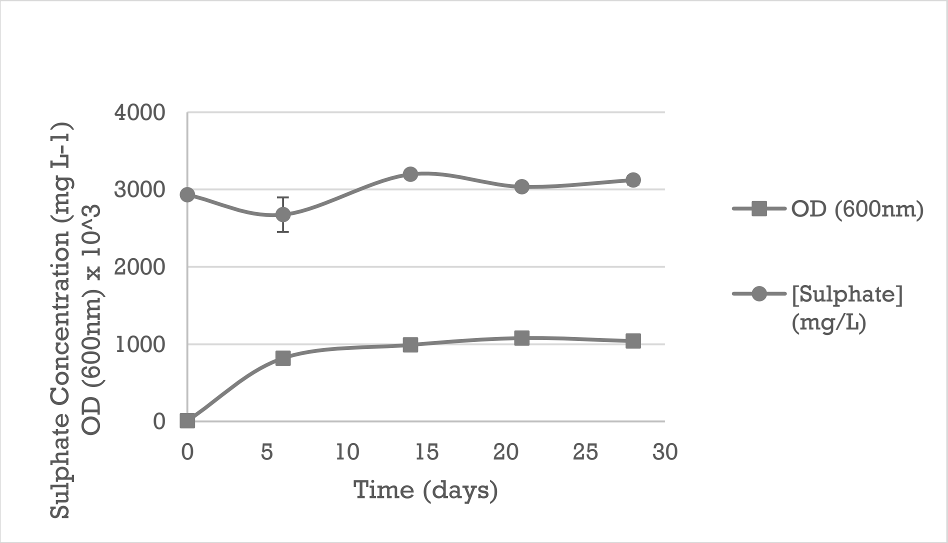

Figure S1 - Performance of bacterial growth monitored by Optical Density (OD) and SRB activity monitored by sulfate concentration evolution in cultures grown in modified Postgate C without palladium. Values are averages ± standard deviations of two replicates (in most cases standard deviations bars are smaller than symbols, therefore not visible).

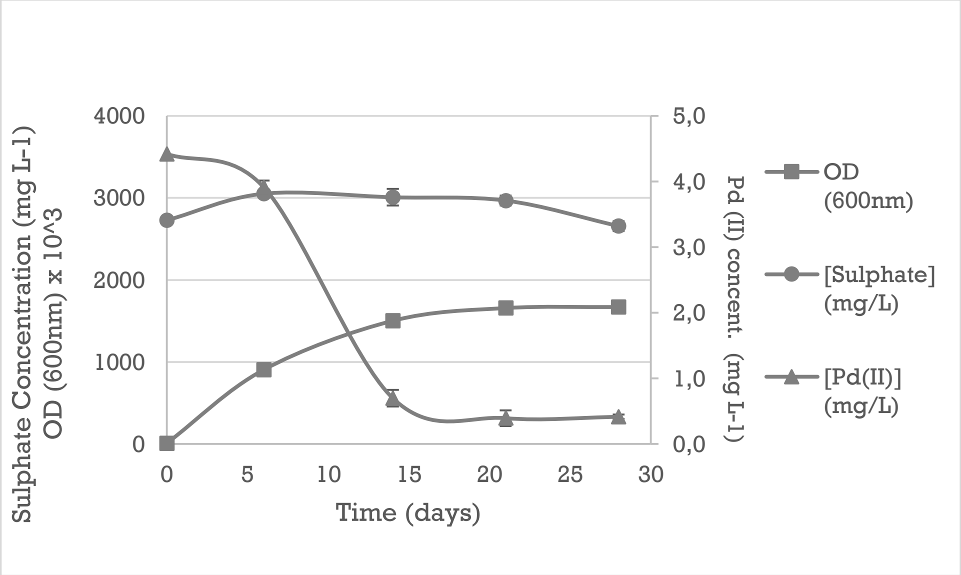

Figure S2 - Evolution of palladium (II) concentration, performance of bacterial growth monitored by Optical Density (OD) and SRB activity monitored by sulfate concentration in cultures grown in modified Postgate C medium with 5 mg L^{-1}

palladium (II). Values are averages ± standard deviations of four replicates (in most cases standard deviations bars are smaller than the symbols, therefore not visible).

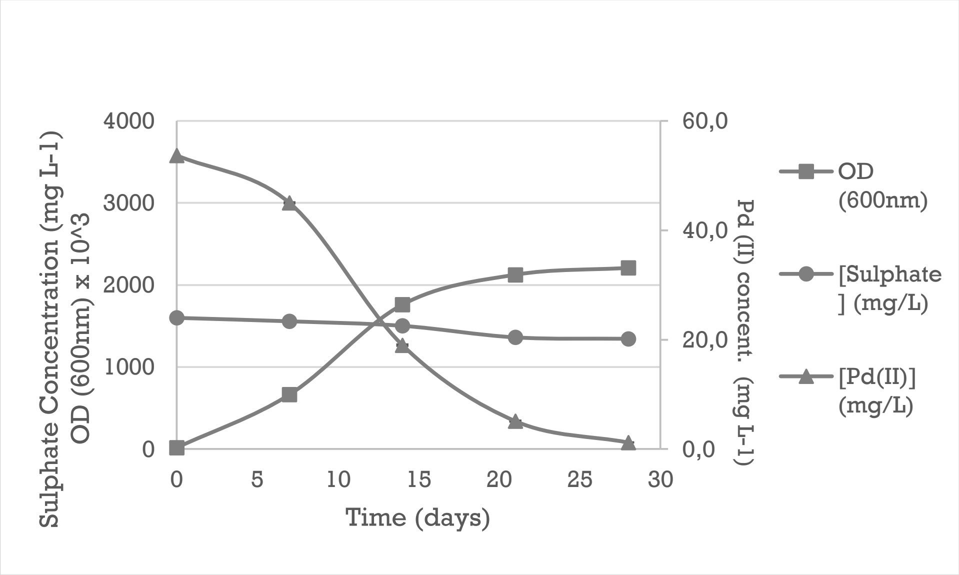

Figure S3 - Evolution of palladium (II) concentration, performance of bacterial growth monitored by Optical Density (OD) and SRB activity monitored by sulfate concentration in cultures grown in modified Postgate C mdium with 50 mg L^{-1} palladium (II). Values are averages ± standard deviations of four replicates (in most cases standard deviations bars are smaller than the symbols, therefore not visible).

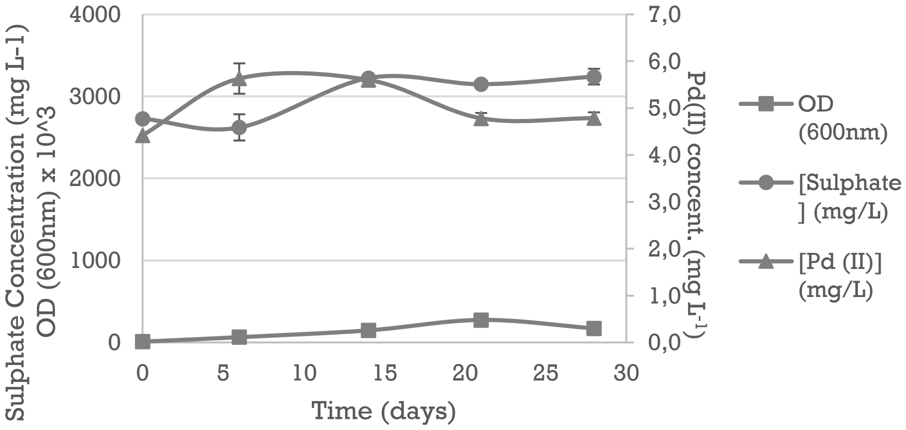

Figure S4 – Evolution of palladium (II) concentration, Optical Density (OD) and sulfate concentration as a function of time on the abiotic control in medium with 5 mg L^{-1} palladium (II). Values are averages ± standard deviations of two replicates (in most cases standard deviations bars are smaller than the symbols, therefore not visible).

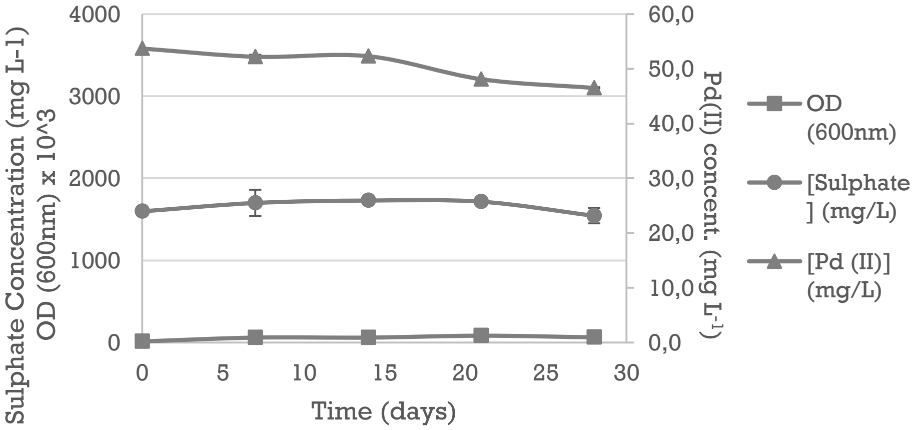

Figure S5 – Evolution of palladium (II) concentration, Optical Density (OD) and sulfate concentration on the abiotic control in medium with 50 mg L^{-1} palladium (II). Values are averages ± standard deviations of two replicates (in most cases standard deviations bars are smaller than the symbols, therefore not visible).

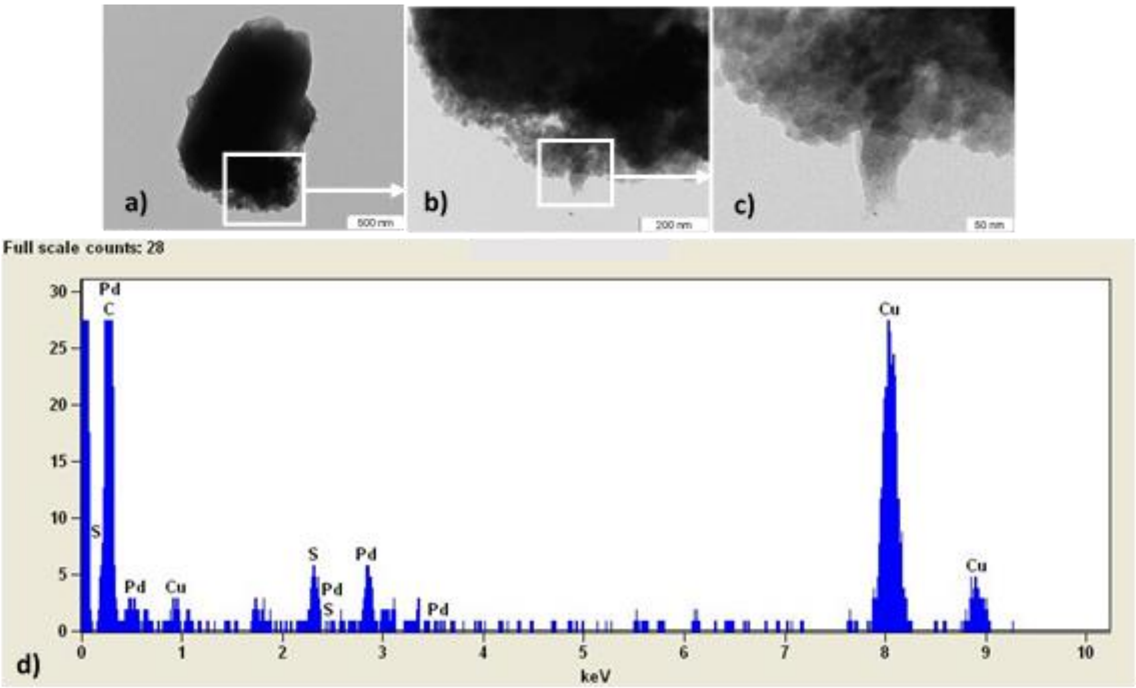

Figure S6 – TEM of a bacterial cell with precipitates from a culture grown in modified Postgate C medium with 50 mg L^{-1} palladium (II), without staining (a, b and c) and EDS spectrum of the precipitates (d).

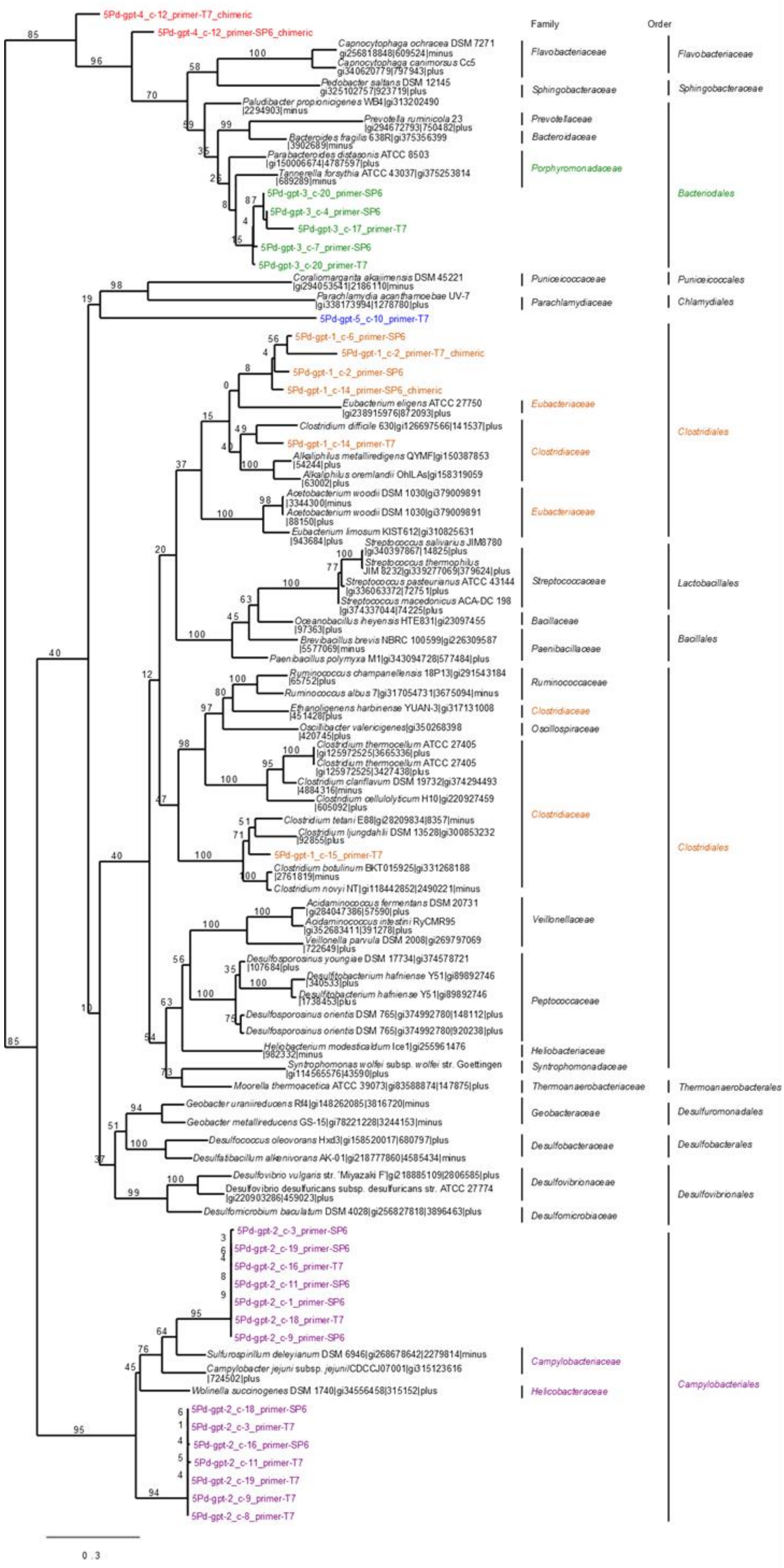
Family
Order
85
5Pd-gpt-4_c-12_primer-T7_chimeric
96
5Pd-gpt-4_c-12_primer-SP6_chimeric
100
Capnocytophaga ochracea DSM 7271 gi256818848|609524|minus
Capnocytophaga canimorsus Cc5 gi340620779|797943|plus
Flavobacteriaceae
Flavobacteriaceae
58
70
Pedobacter saltans DSM 12145 gi325102757|923719|plus
Sphingobacteraceae
Sphingobacteraceae
Paludibacter propionicigenes WB4|gi313202490 |2294903|minus
59
99
Prevotella ruminicola 23 |gi294672793|750482|plus
Prevotellaceae
Bacteroides fragilis 638R|gi375356399 |3902689|minus
Bacteroidaceae
35
Parabacteroides distasonis ATCC 8503 |gi150006674|4787597|plus
26
Tannerella forsythia ATCC 43037|gi375253814 |689289|minus
Porphyromonadaceae
8
87
5Pd-gpt-3_c-20_primer-SP6
4
5Pd-gpt-3_c-4_primer-SP6
5Pd-gpt-3_c-17_primer-T7
15
5Pd-gpt-3_c-7_primer-SP6
5Pd-gpt-3_c-20_primer-T7
Bacteriodales
19
98
Coraliomargarita akajimensis DSM 45221 |gi294053541|2186110|minus
Puniceicoccaceae
Puniceicoccales
Parachlamydia acanthamoebae UV-7 |gi338173994|1278780|plus
Parachlamydiaceae
Chlamydiales
5Pd-gpt-5_c-10_primer-T7
56
5Pd-gpt-1_c-6_primer-SP6
4
5Pd-gpt-1_c-2_primer-T7_chimeric
8
5Pd-gpt-1_c-2_primer-SP6
0
5Pd-gpt-1_c-14_primer-SP6_chimeric
Eubacterium eligens ATCC 27750 |gi238915976|872093|plus
Eubacteriaceae
15
49
Clostridium difficile 630|gi126697566|141537|plus
40
5Pd-gpt-1_c-14_primer-T7
Clostridiaceae
Clostridiales
100
Alkaliphilus metalliredigens QYMF|gi150387853 |54244|plus
Alkaliphilus oremlandii OhILAs|gi158319059 |63002|plus
37
98
Acetobacterium woodii DSM 1030|gi379009891 |3344300|minus
100
Acetobacterium woodii DSM 1030|gi379009891 |88150|plus
Eubacteriaceae
Eubacterium limosum KIST612|gi310825631 |943684|plus
20
100
Streptococcus salivarius JIM8780 |gi340397867|14825|plus
77
Streptococcus thermophilus JIM 8232|gi339277069|379624|plus
100
Streptococcus pasteurianus ATCC 43144 |gi336063372|72751|plus
Streptococcaceae
Lactobacillales
63
Streptococcus macedonicus ACA-DC 198 |gi374337044|74225|plus
45
Oceanobacillus iheyensis HTE831|gi23097455 |97363|plus
Bacillaceae
100
Brevibacillus brevis NBRC 100599|gi226309587 |5577069|minus
Bacillales
Paenibacillaceae
Paenibacillus polymyxa M1|gi343094728|577484|plus
40
12
100
Ruminococcus champanellensis 18P13|gi291543184 |65752|plus
Ruminococcaceae
80
Ruminococcus albus 7|gi317054731|3675094|minus
97
Ethanoligenens harbinense YUAN-3|gi317131008 |451428|plus
Clostridiaceae
Oscillibacter valericigenes|gi350268398 |420745|plus
Oscillospiraceae
98
100
Clostridium thermocellum ATCC 27405 |gi125972525|3665336|plus
95
Clostridium thermocellum ATCC 27405 |gi125972525|3427438|plus
100
Clostridium clariflavum DSM 19732|gi374294493 |4884316|minus
Clostridium cellulolyticum H10|gi220927459 |605092|plus
Clostridiaceae
47
51
Clostridium tetani E88|gi28209834|8357|minus
71
Clostridium ljungdahlii DSM 13528|gi300853232 |92855|plus
Clostridiales
40
100
5Pd-gpt-1_c-15_primer-T7
Clostridium botulinum BKT015925|gi331268188 |2761819|minus
100
Clostridium novyi NT|gi118442852|2490221|minus
100
Acidaminococcus fermentans DSM 20731 |gi284047386|57590|plus
100
Acidaminococcus intestini RyCMR95 |gi352683411|391278|plus
Veillonellaceae
Veillonella parvula DSM 2008|gi269797069 |722649|plus
56
Desulfosporosinus youngiae DSM 17734|gi374578721 |107684|plus
35
100
Desulfitobacterium hafniense Y51|gi89892746 |340533|plus
100
Desulfitobacterium hafniense Y51|gi89892746 |1738453|plus
Peptococcaceae
10
63
75
Desulfosporosinus orientis DSM 765|gi374992780|148112|plus
Desulfosporosinus orientis DSM 765|gi374992780|920238|plus
85
54
Heliobacterium modesticaldum Ice1|gi255961476 |982332|minus
Heliobacteriaceae
73
Syntrophomonas wolfei subsp. wolfei str. Goettingen |gi114565576|43590|plus
Syntrophomonadaceae
Moorella thermoacetica ATCC 39073|gi83588874|147875|plus
Thermoanaerobacteriaceae
Thermoanaerobacterales
94
Geobacter uraniireducens Rf4|gi148262085|3816720|minus
Geobacteraceae
Desulfuromonadales
51
Geobacter metallireducens GS-15|gi78221228|3244153|minus
100
Desulfococcus oleovorans Hxd3|gi158520017|680797|plus
Desulfobacteraceae
Desulfobacterales
37
Desulfatibacillum alkenivorans AK-01|gi218777860|4585434|minus
100
Desulfovibrio vulgaris str. 'Miyazaki F'|gi218885109|2806585|plus
Desulfovibrionaceae
99
Desulfovibrio desulfuricans subsp. desulfuricans str. ATCC 27774 |gi220903286|459023|plus
Desulfovibrionales
Desulfomicrobium baculatum DSM 4028|gi256827818|3896463|plus
Desulfomicrobiaceae
3
5Pd-gpt-2_c-3_primer-SP6
6
5Pd-gpt-2_c-19_primer-SP6
4
5Pd-gpt-2_c-16_primer-T7
8
5Pd-gpt-2_c-11_primer-SP6
9
5Pd-gpt-2_c-1_primer-SP6
95
5Pd-gpt-2_c-18_primer-T7
5Pd-gpt-2_c-9_primer-SP6
64
76
Sulfurospirillum deleyianum DSM 6946|gi268678642|2279814|minus
Campylobacteriaceae
45
Campylobacter jejuni subsp. jejuniICDCCJ07001|gi315123616 |724502|plus
Wolinella succinogenes DSM 1740|gi34556458|315152|plus
Helicobacteraceae
Campylobacteriales
6
5Pd-gpt-2_c-18_primer-SP6
1
5Pd-gpt-2_c-3_primer-T7
95
4
5Pd-gpt-2_c-16_primer-SP6
5
5Pd-gpt-2_c-11_primer-T7
4
5Pd-gpt-2_c-19_primer-T7
94
5Pd-gpt-2_c-9_primer-T7
5Pd-gpt-2_c-8_primer-T7
0.3

Figure S7 - Phylogenetic tree constructed with the 16S rRNA partial gene sequences obtained for the consortium grown with 5 mg L^{-1} Pd and a set of 57 16S rRNA reference sequences, using the BIONJ algorithm with PhyML 3.0 algorithms, methods and utilities (Guindon et al, 2010). The GTR substitution model was used with a proportion of invariable sites of 0.29 and a gamma distribution parameter of 0.73. Reliability for internal branching was assessed using 100 bootstrap replicates. Bootstrap values are on the left side of respective cluster node.

Table S1 - Order, Family and Genus taxonomic classifications retrieved by RDP classifier for the 16S rRNA gene sequences obtained for community grown with 5 mg L^{-1} palladium (II).

Gel pattern type	Clones sequenced	Sequencing primer	NCBI accession ¹	Covered hypervariable regions	Chimera detected	Order	Conf.	Family	Conf.	Genus	Conf.
5Pd-gpt-1	5Pd-c-2	SP6	KT452874	V5, V6, V7, V8, V9		*Clostridiales*	91%	*Eubacteriaceae*	26%	*Alkalibacter*	24%
	5Pd-c-2	T7	U-LB	V1, V2, V3, V4 (65%)	Yes	*Cytophagales*	17%	*Flammeovirgaceae*	17%	*Flexithrix*	17%
	5Pd-c-6	SP6	KT452872	V2 (91%), V3, V4		*Clostridiales*	100 %	*Clostridiaceae* 3	43%	*Clostridiisalibacter*	32%
	5Pd-c-14	SP6	KT452875	V5 (51%), V6, V7, V8, V9		*Clostridiales*	99%	*Peptostreptococcaceae*	88%	*Clostridium* XI	61%
	5Pd-c-14	T7	U-LB	V1, V2, V3, V4	Yes	*Clostridiales*	100 %	*Peptostreptococcaceae*	69%	*Clostridium* XI	64%
	5Pd-c-15	T7	KT452873	V1, V2, V3, V4		*Clostridiales*	100 %	*Clostridiaceae* 1	100%	*Clostridium* sensu stricto	100%
5Pd-gpt-2	5Pd-c-1	SP6	KT452865	V1, V2, V3, V4		*Campylobacterales*	100 %	*Campylobacteraceae*	100%	*Arcobacter*	100%
	5Pd-c-3	SP6	KT452866	V1, V2, V3, V4 (40%)		*Campylobacterales*	100 %	*Campylobacteraceae*	100%	*Arcobacter*	100%
	5Pd-c-3	T7	KT452884	V5, V6, V7, V8, V9		*Campylobacterales*	100 %	*Campylobacteraceae*	100%	*Arcobacter*	100%
	5Pd-c-8	T7	KT452883	V5, V6, V7, V8, V9		*Campylobacterales*	100 %	*Campylobacteraceae*	100%	*Arcobacter*	100%
	5Pd-c-9	SP6	KT452871	V1, V2, V3, V4, V5		*Campylobacterales*	100 %	*Campylobacteraceae*	100%	*Arcobacter*	100%
	5Pd-c-9	T7	KT452882	V5, V6, V7, V8, V9		*Campylobacterales*	100 %	*Campylobacteraceae*	100%	*Arcobacter*	100%
	5Pd-c-11	SP6	KT452870	V1, V2, V3, V4		*Campylobacterales*	100 %	*Campylobacteraceae*	100%	*Arcobacter*	100%
	5Pd-c-11	T7	KT452885	V6 (20%), V7, V8, V9		*Campylobacterales*	100 %	*Campylobacteraceae*	100%	*Arcobacter*	100%
	5Pd-c-16	SP6	KT452880	V5, V6, V7, V8, V9		*Campylobacterales*	100 %	*Campylobacteraceae*	100%	*Arcobacter*	100%

	5Pd-c-16	T7	KT452869	V1, V2, V3, V4		*Campylobacterales*	100%	*Campylobacteraceae*	100%	*Arcobacter*	100%
	5Pd-c-18	SP6	KT452879	V5, V6, V7, V8, V9		*Campylobacterales*	100%	*Campylobacteraceae*	100%	*Arcobacter*	100%
	5Pd-c-18	T7	KT452867	V1, V2, V3, V4 (91%)		*Campylobacterales*	100%	*Campylobacteraceae*	100%	*Arcobacter*	100%
	5Pd-c-19	SP6	KT452868	V5, V6, V7, V8, V9		*Campylobacterales*	100%	*Campylobacteraceae*	100%	*Arcobacter*	100%
	5Pd-c-19	T7	KT452881	V1, V2, V3, V4 (86%)		*Campylobacterales*	100%	*Campylobacteraceae*	100%	*Arcobacter*	100%
5Pd-gpt-3	5Pd-c-4	SP6	KT452877	V5, V6, V7, V8, V9		*Bacteroidales*	100%	*Porphyromonadaceae*	100%	*Parabacteroides*	100%
	5Pd-c-7	SP6	KT452863	V1, V2, V3, V4		*Bacteroidales*	100%	*Porphyromonadaceae*	100%	*Parabacteroides*	97%
	5Pd-c-17	T7	U-LB	V5, V6, V7, V8, V9	Yes	*Bacteroidales*	100%	*Porphyromonadaceae*	100%	*Parabacteroides*	83%
	5Pd-c-20	SP6	KT452878	V5, V6, V7, V8, V9		*Bacteroidales*	100%	*Porphyromonadaceae*	100%	*Parabacteroides*	100%
	5Pd-c-20	T7	KT452864	V1, V2, V3, V4 (82%)		*Bacteroidales*	100%	*Porphyromonadaceae*	100%	*Parabacteroides*	96%
5Pd-gpt-4	5Pd-c-12	SP6	U-LB	V1, V2, V3, V4	Yes	*Campylobacterales*	63%	*Campylobacteraceae*	63%	*Arcobacter*	47%
	5Pd-c-12	T7	U-LB	V5, V6, V7, V8, V9	Yes	*Bacteroidales*	100%	*Porphyromonadaceae*	99%	*Proteiniphilum*	93%
5Pd-gpt-5	5Pd-c-10	T7	KT452876	V5, V6, V7, V8, V9		*Spirochaetales*	100%	*Spirochaetaceae*	100%	*Sphaerochaeta*	100%

1 - U-LB means Unpublished - Listed Below

Table S2 - Order, Family and Genus taxonomic classifications retrieved by RDP classifier for the 16S rRNA gene sequences obtained for consortium grown with 50 mg L^{-1} palladium (II).

Gel pattern type	Clones sequenced	Sequencing primer	NCBI accession	Hypervariable regions covered	Chimera detected	Order	Conf.	Family	Conf.	Genus	Conf.
50Pd-gpt-1	50Pd-c-19	T7	KT452887	V1, V2, V3, V4		*Sphingobacteriales*	32%	*Sphingobacteriaceae*	31%	*Solitalea*	18%
50Pd-gpt-2	50Pd-c-17	T7	KT452889	V5, V6, V7, V8, V9		*Clostridiales*	100%	*Ruminococcaceae*	100%	*Hydrogenoanaerobacterium*	64%
50Pd-gpt-3	50Pd-c-20	T7	KT452888	V5, V6, V7, V8, V9		*Clostridiales*	100%	*Clostridiaceae* 1	100%	*Clostridium* sensu stricto	100%
50Pd-gpt-4	50Pd-c-30	T7	KT452886	V1, V2, V3, V4		*Clostridiales*	100%	*Lachnospiraceae*	100%	*Clostridium* XlVa	99%
50Pd-gpt-5	50Pd-c-9	T7	U-LB	V5, V6, V7, V8, V9	Yes	*Clostridiales*	100%	*Clostridiaceae* 2	100%	*Alkaliphilus*	95%
50Pd-gpt-6	50Pd-c-16	T7	U-LB	V1, V2, V3, V4 (16%)	Yes	*Clostridiales*	100%	*Clostridiaceae* 1	100%	*Clostridium sensu stricto*	94%

1 - U-LB means Unpublished - Listed Below

Table S3 - Order, Family and Genus taxonomic classifications retrieved by RDP classifier for the 16S rRNA gene sequences obtained for consortium grown without palladium (II).

Gel pattern type	Clones sequenced	Sequencing primer	NCBI accession	Hypervariable regions covered	Chimera detected	Order	Conf.	Family	Conf.	Genus	Conf.
noPd-gpt-1	noPd-c-17	T7	U-LB	V5, V6, V7, V8, V9	Yes	*Clostridiales*	100%	*Ruminococcaceae*	99%	*Oscillibacter*	96%
noPd-gpt-2	noPd-c-4	T7	KT452894	V1, V2, V3, V4		*Sphingobacteriales*	21%	*Sphingobacteriaceae*	20%	*Arcticibacter*	15%
noPd-gpt-3	noPd-c-18	T7	KT452896	V1, V2, V3, V4		*Bacteroidales*	100%	*Porphyromonadaceae*	100%	*Parabacteroides*	99%
noPd-gpt-4	noPd-c-14	T7	KT452892	V5, V6, V7, V8, V9		*Bacteroidales*	100%	*Porphyromonadaceae*	100%	*Proteiniphilum*	74%
noPd-gpt-5	noPd-c-28	T7	KT452890	V5, V6, V7, V8, V9		*Bacteroidales*	93%	*Porphyromonadaceae*	86%	*Proteiniphilum*	59%

noPd-gpt-6	noPd-c-22	T7	KT452893	V5, V6, V7, V8, V9	*Clostridiales*	99%	*Clostridiaceae* 1	65%	*Fervidicella*	52%
noPd-gpt-7	noPd-c-15	T7	KT452891	V6, V7, V8, V9	*Bacteroidales*	59%	*Marinilabiliaceae*	34%	*Alkalitalea*	22%
noPd-gpt-8	noPd-c-19	T7	KT452895	V1, V2, V3, V4	*Clostridiales*	100%	*Clostridiaceae* 3	55%	*Clostridiisalibacter*	37%

1 - U-LB means Unpublished - Listed Below

Unpublished putative chimeric sequences

>5Pd-c-2_T7

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APPENDIX 2

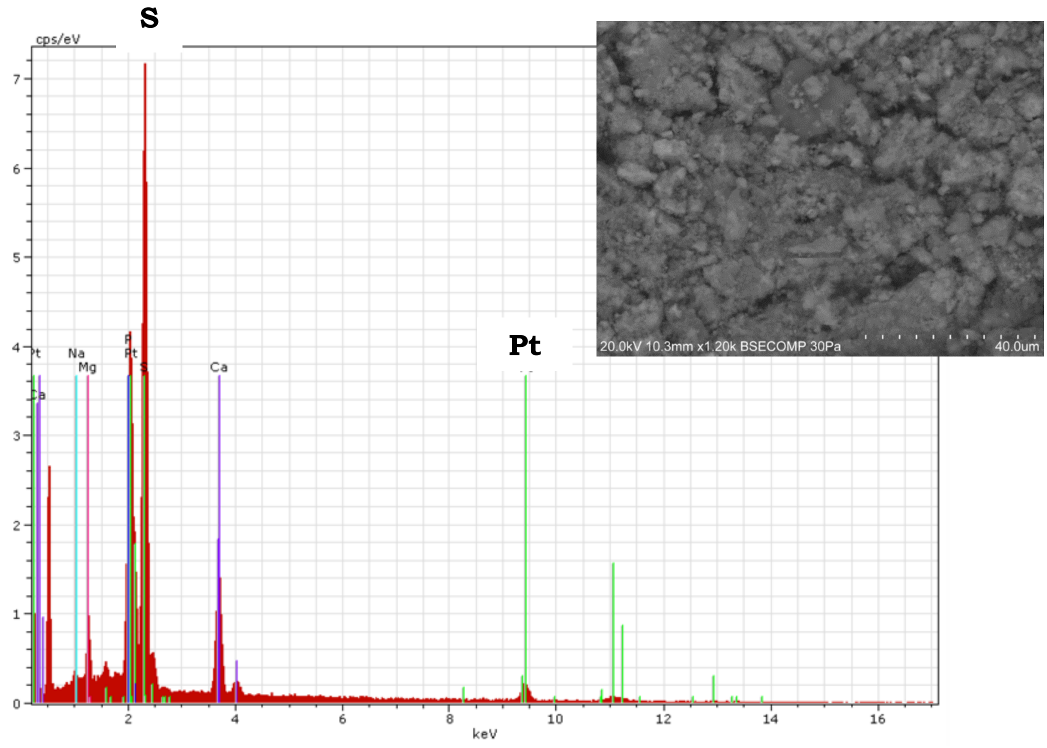


Figure S8 - SEM image and EDS spectrum of the precipitates obtained in the *batch* assays in which the platinum loaded stripping phase (seawater) was added to the metabolic products.

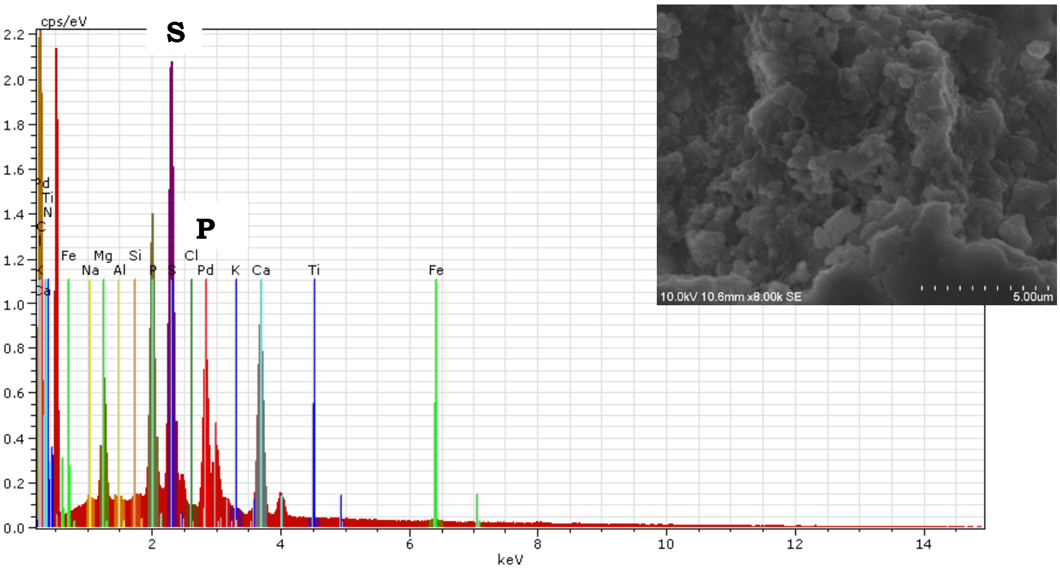


Figure S9 - SEM image and EDS spectrum of the precipitates obtained in the *batch* assays in which the palladium loaded stripping phase (seawater) was added to the metabolic products.

APPENDIX 3

1. Methods used in the analysis and characterization of the biorecovered precipitates

The analysis and characterization of the biorecovered precipitates is extremely important, allowing to determine the percentage of metal recovered and characterizing the metals particles, regarding to, elemental composition, morphology, structure, size, and other properties that may contribute for testing these particles for several applications.

1.1. Methods used for metals analysis

The determination of the concentration of the elements in precipitates is performed by quantitative chemical analysis. Several analysis techniques are used to quantify the presence of metals in a sample and that determination can comprise one or more complementary techniques, which can include atomic absorption spectrometry (AAS), spark optical emission spectroscopy (Spark OES), inductively coupled plasma spectroscopy (ICP), X-ray fluorescence spectroscopy (XRF), wet chemical analyses, combustion methods, and inert gas fusion (IG) (MEE, 2014; Gennaro *et al.*, 2015). The choice of a specific technique will depend on the type of sample, the amount of material for analysis, the intended results, the equipment availability and the analytical cost (MEE, 2014). The analytical techniques most used nowadays to quantify metals in an aqueous sample are AAS and ICP.

AAS is an analytical technique that measures the concentrations of individual elements in an aqueous sample and can analyse more than 62 different metals. Flame-AAS hasusually a sensitivity of parts per million, but it can go below of parts per billion of a gram (ug.dm^{-3}) in a sample if graphite furnace is used (Gennaro *et al.*, 2015).

The principle of AAS is the use of light specifically absorbed by an element, thus, the atoms of each element absorb characteristic wavelengths of light. The sample is converted into ground state free atoms in the vapour state, meaning that the sample is atomized and the excited atoms emit a beam of electromagnetic radiation that pass through the vaporised sample. Some of the radiation is absorbed by the atoms in the sample (Gennaro *et al.*, 2015).

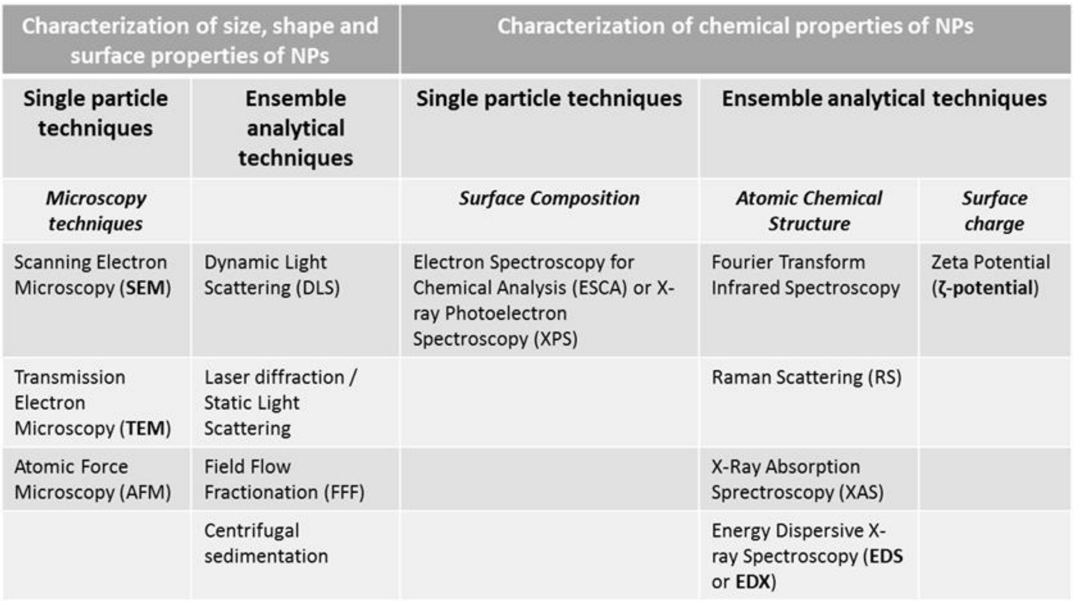
Techniques of ICP have been used to detect and analyze simultaneously trace (ppb-ppm) and ultra-trace (ppq-ppb) elements in a sample. There are several ICP techniques, such as Inductively Coupled Plasma – Atomic Emission Spectrometry (ICP-AES), Inductively coupled plasma - Optical Emission Spectroscopy (ICP-OES) and Inductively Coupled Plasma – Mass Spectrometry (ICP-MS).

From all of these ICP, the ICP-MS is the technique most widely used for the determination of metals concentration. ICP-MS provides the necessary accurate and precise measurements, providing low detection limits and has been used for the determination of metals concentration (Inductively Coupled Plasma (ICP), 2016). In this technique the ions pass through a series of apertures (cones) into the high vacuum mass analyser. The mass-to-charge ratio (m/e) of the elements identify their isotopes and the intensity of a specific peak in the mass spectrum is proportional to the quantity of that isotope in the original sample (Gennaro *et al.*, 2015).

1.2. Characterization techniques of metal precipitates

The search and development of new methods of materials preparation have gain importance in the last years and thus the characterization of such materials is extremely important. All the new material are characterized by advances techniques, such as scanning electron microscopy (SEM), transmission electron microscopy (TEM) and atom mapping techniques, X-Ray Diffraction (XRD), X-Ray Photoelectron Spectroscopy (XPS) and X-ray absorption Spectroscopy (XAS). Nanotechnology also focused in the nanomaterials characterization and analytical techniques available, as such those shown in Table S4 (Gabor *et al.*, 2008).

Table S4 – Characterization of metal NPs (adapted from Nath and Banerjee, 2013)



Characterization of size, shape and surface properties of NPs		Characterization of chemical properties of NPs		
Single particle techniques	**Ensemble analytical techniques**	**Single particle techniques**	**Ensemble analytical techniques**	
Microscopy techniques		***Surface Composition***	***Atomic Chemical Structure***	***Surface charge***
Scanning Electron Microscopy (**SEM**)	Dynamic Light Scattering (DLS)	Electron Spectroscopy for Chemical Analysis (ESCA) or X-ray Photoelectron Spectroscopy (XPS)	Fourier Transform Infrared Spectroscopy	Zeta Potential (**ζ-potential**)
Transmission Electron Microscopy (**TEM**)	Laser diffraction / Static Light Scattering		Raman Scattering (RS)	
Atomic Force Microscopy (AFM)	Field Flow Fractionation (FFF)		X-Ray Absorption Sprectroscopy (XAS)	
	Centrifugal sedimentation		Energy Dispersive X-ray Spectroscopy (**EDS** or **EDX**)	

From the different analysing techniques that can be applied in nanoparticles characterization are here introduced only those which were used in the present work.

XRD

X-ray diffraction is a very important characterization technique used in solid state chemistry and materials science (Loye, 2013). The XRD pattern features information about crystalline structure, grain size and strain (Singh and Chauhan, 2009).

XRD has been applied mainly in two approaches: fingerprint characterization of crystalline materials and determination of their structure.

Fingerprint characterization of crystalline materials is possible because each crystalline solid has single and characteristic X-ray powder pattern.

For the structure determination, the X-ray crystallography can be used. This determination allows to understand, for instance, how the atoms pack together in the crystalline state, the interatomic distance and angle (Loye, 2013).

The XRD patterns of a sample have to be compared with a standard XRD pattern sheet, as shown in the example in Figure S10.

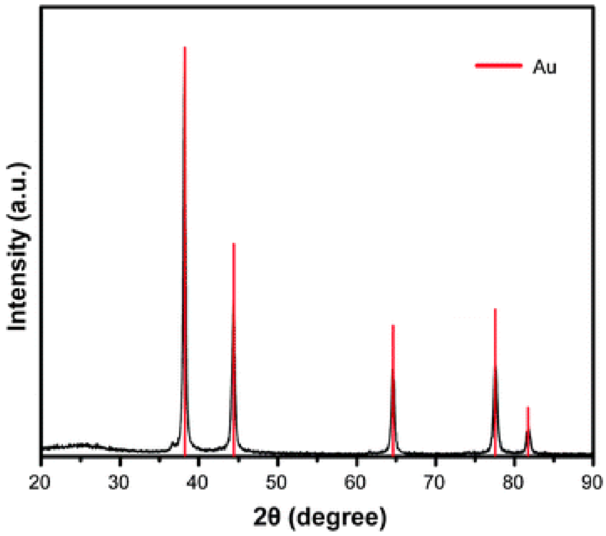


Figure S10 - XRD pattern (black) of Au/C synthesized compared with the standard Au XRD PDF# 00-004-0784 (red). (Adapted from Guo and Suslick, 2012)

In this example, XRD confirms the crystallinity of the Au NPs in an amorphous carbon matrix (Guo and Suslick, 2012).

However, the XRD signal of nanometric particles and of poorly crystallized materials is normally week and presents elongated and poorly defined diffraction peaks, thus it can be difficult to extract quantitative information from the diffraction patterns of nanosized and poorly crystallized compounds (Sugimoto, 2001; Haruta *et al.*, 1998).

Several approaches have been used to analyse the XRD line profiles, as Scherrer, Williamson–Hall and Warren–Averbach methods (Scherrer, 1918; Williamson and Hall, 1953; Warren and Averbach, 1952; Warren, 1969). Each one of these methods possess different information values, reliabilities of results obtained, and calculation laboriousness. Researchers have had difficulty in selecting one of them for the particles analysis and in most methods the average particle size is determined. The particle size average can be performed over the radius, surface area, specific surface area, or volume of a material, depending on the method used and the average values obtained by each method may be significantly different (Dorofeev *et al.*, 2012).

Electron Microscopy

SEM, one of the most used technique for the characterization of nanostructures, is a microscope that form an image using electrons instead of light. SEM presents advantages comparing to traditional microscopes, such as, the large depth of field, the higher resolution (Suresh, 2015).

TEM has been used to determine the morphology and structure of the NPs composite materials. TEM can also provide information about the particle size and size distribution of the NPs (Akbari *et al.*, 2011). The particles size are usually calculated and expressed as the diameter of a sphere that has the same projected area as the projected image of the particle. For the particle size determination two techniques are used, manual or automatic. The manual technique is usually based on the measurement using a marking device moved along the particle to obtain its linear dimensional measure. A considerable number of particles in the sample should be measured and divided by the number of particles to get a mean result (Jillavenkatesa *et al.*, 2001). TEM can also give information about the dispersion / agglomeration of the particles in a sample (Akbari *et al.*, 2011).

The difference between SEM and TEM images can be shown in Figure S11. In SEM micrograph, Figure S11-A, it is possible to have information about particles morphology, while in TEM micrograph, Figure S11-B, beside particles morphology, it is also possible to know about, for example, particles size and particles distribution. TEM is usually used in higher magnifications than SEM, as it shown in the scales of Figure S11. In SEM, the material surface is observed and in backscatter mode the observation of the compositional differences (and with EDS obtain chemical differences) can be possible. In TEM, an image close to atomical structure may be possible and it is obtained crystallographic information.

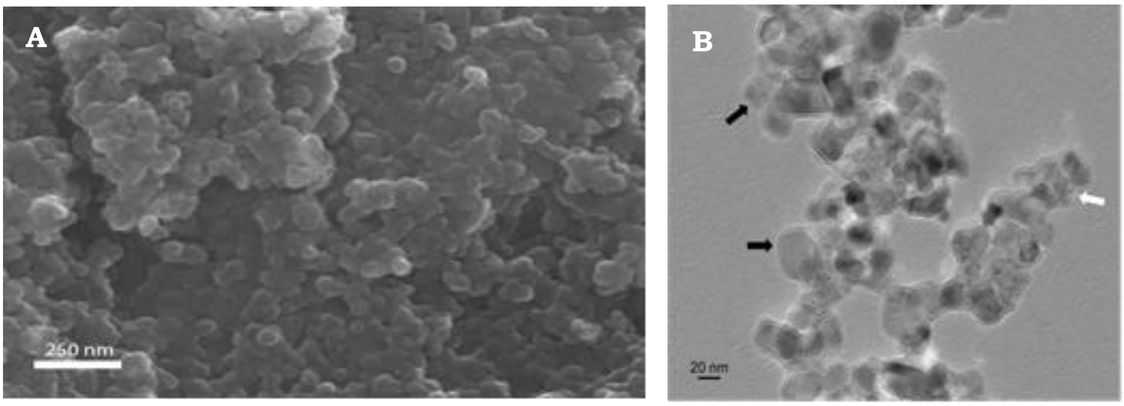


Figure S11 – SEM (**A**) and TEM (**B**) images of the nanosized particles of ZnS (white arrows) decorating TiO_2 (black arrows) (adapted from da Costa *et al.*, 2012)

Electron microscopy, as SEM and TEM, in combination with diffraction studies, for instance coupled to energy dispersive X-ray spectroscopy (EDS), becomes a very valuable aid for elemental nanoparticles characterization (Rawle, 2002).

EDS

EDS, also referred as EDX, provides elemental analysis of a sample inside a SEM or TEM, since it allows identifying the elements and their relative proportions in the sample.

EDS involves a localized chemical analysis using a characteristic X-ray spectrum emitted by a solid sample bombarded with an electrons beam. This technique can detect all elements from atomic number 4 (Be) to 92 (U) (http://cfamm.ucr.edu/documents/eds-intro.pdf). The characteristic x-ray energies emitted for elements is usually different between elements and only a few spectral peaks overlapping (http://www.andersonmaterials.com/edx-eds.html). In Figure S12, different elements of a nanocomposite of ZnS/TiO_2, such as, as Zn, S and Ti, were identified in a SEM micrograph by EDS.

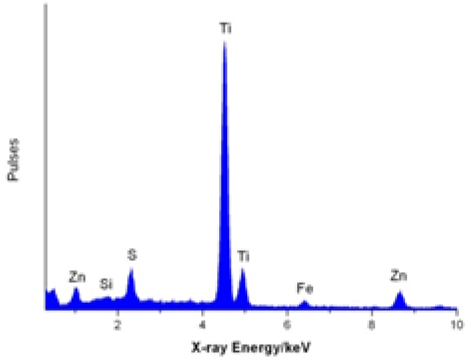
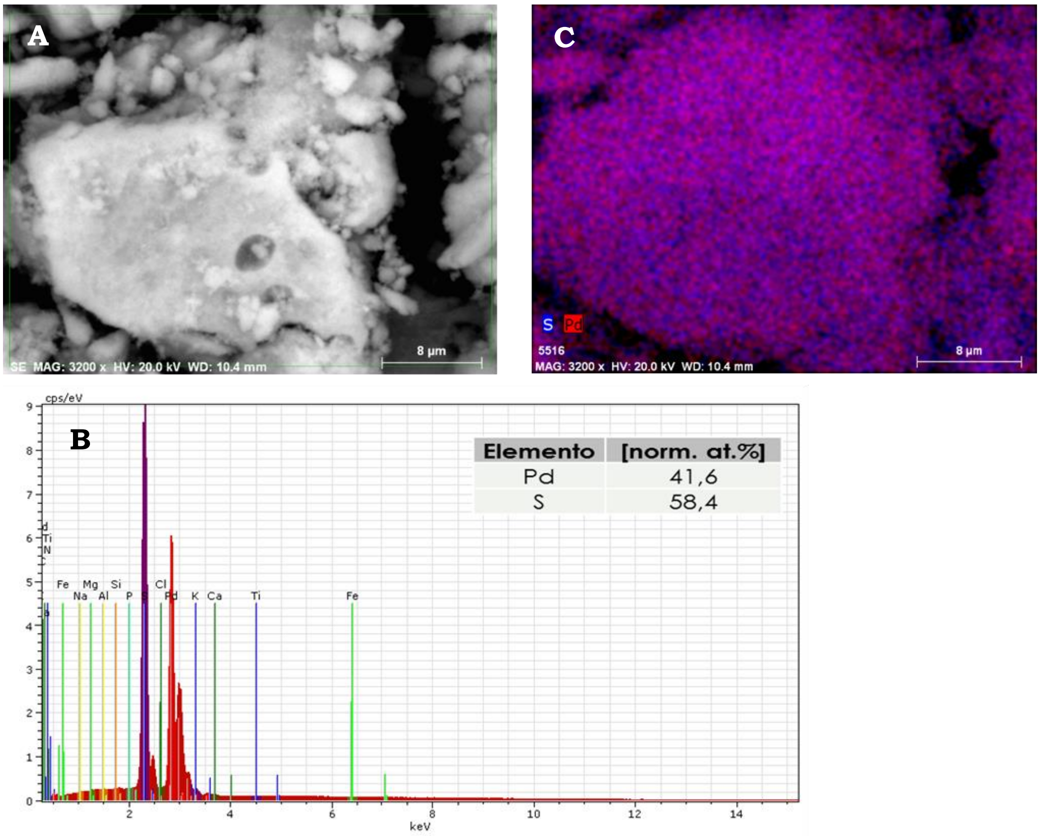


Figure S12 – EDS spectra obtained from the SEM image in figure X A, of the nanosized particles of nanocomposite ZnS/TiO_2 (adapted from da Costa *et al.*, 2012)

In EDS the qualitative analysis is based on the identification of the lines in the EDS spectrum, while quantitative analysis involves the measurement of the line intensities for each sample element and for the same elements in calibration standards of known composition (http://cfamm.ucr.edu/documents/eds-intro.pdf).

The sample, for example in SEM, can be scanned and the intensity of a selected X-ray line can be displayed and element distribution images or 'maps' can be produced (http://cfamm.ucr.edu/documents/eds-intro.pdf). As an example, a sample analysed by SEM coupled to EDS and respective mapping of the elements is shown, in Figure S13.



Elemento	[norm. at.%]
Pd	41,6
S	58,4

Figure S13 – SEM image (**A**) and respective EDS spectrum (**B**) and elements mapping (**C**) of a precipitate obtained from a study using an effluent from an AMD bioremediation process for Pd(II) recovery from an aqueous solution.

The mapping elements, Figure S13 C, allow to conclude that the particle, analysed by SEM (Figutre S13-A), presents the elements Pd (in red) and S (in blue) overlapped (pink = overlapping of red and blue) and despite the spectrum identified other elements in the sample, the Pd and S have higher intensities. As mentioned before, the EDS analysis (Figure S13-B) also allow to determine the relative proportions of the elements in the sample, and in this case, the precipitate analysed have an atomic percentage of 41.6 and 58.4 for Pd and S, respectively, which corroborates the mapping results.

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About the author

Ana Filipa Benedito da Assunção was born on 16 September 1984 in Faro, Portugal. In December 2007 she completed the Biotechnological Engineering degree and after a year completed the Biological Engineering Mater degree, both at University of Algarve. In 2009, she started her scientific work in a research Project in Águas do Algarve company. The main objective of this project was to evaluate the possibility of bromate (a carcinogenic ion) removal from drinking water, using activated carbon filters with biological activity. From February 2010 to February 2011, in order to continue this research work, she was in a PhD fellow at Águas do Algarve, funded by Foundation for Science and Technology (FCT) (that fellow was suspended due to the economical situation of the company). In the same year Ana worked in a Solvent Extraction Project based on the PGM extraction by organic solvents. In January 2012, she iniciate her PhD fellow at the EcoReach Group from CCMAR, University of Algarve, funded by Foundation for Science and Technology (FCT). The results obtained during this PhD are shown in the present thesis.