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"Microbial reduction of palladium: mechanisms, modeling and applications in wastewater treatment systems"

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Aurora Margarita Pat Espadas

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Dr. Francisco J. Cervantes Carrillo

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Dedicado a:

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Aurora M. Pat-Espadas. 2015**. "Reducción microbiana de paladio: mecanismos, modelado y aplicaciones en sistemas de tratamiento de aguas".** Tesis Doctoral, IPICYT, San Luis Potosí, México

El potencial metabólico que poseen los microorganismos de cambiar el estado de oxidación de los metales es ampliamente conocido y constituye una herramienta poderosa que se puede emplear en el desarrollo y aplicación de nuevas técnicas de biorremediación. En la actualidad, no sólo la recuperación de metales sino también la producción de nanopartículas metálicas es un tema de interés y las propuestas de enfoque biotecnológico han surgido como opciones prometedoras para alcanzar este propósito.

El paladio (Pd) es considerado como un metal precioso con propiedades excepcionales lo cual lo hace atractivo para aplicarlo en muchas áreas de la industria, aumentando su uso y demanda. Sin embargo, su abundancia en la tierra es limitada y los esfuerzos por recuperarlo cuando se encuentra presente en efluentes y residuos es mínimo. Esta tesis se enfoca en ofrecer una solución a esta problemática, se propone un método biológico para la recuperación de Pd y producción de nanopartículas de Pd(0).

Para lograr tal objetivo se evaluaron un cultivo puro de *Geobacter sulfurreducens,* cepa PCA y lodo granular metanogénico a fin de entender los mecanismos involucrados.

G. sulfurreducens es un microorganismo con capacidades metabólicas excepcionales, se encuentra ampliamente distribuido en suelo y sedimentos, y su

notable capacidad fisiológica es de gran interés desde el punto de vista biotecnológico. Los resultados obtenidos demostraron, por vez primera, que *G. sulfurreducens* es capaz de acoplar la oxidación de acetato con la reducción de Pd(II) y producir nanopartículas de Pd(0). Los parámetros células:Pd, composición del medio, pH, especiación, concentración de Pd(II) y la presencia de un mediador redox son de relevancia en el proceso. Se demostró que la adición de un mediador redox como la antraquinona-2,6-disulfonato (AQDS), favorece la síntesis extracelular de nanoapartículas de Pd(0), lo cual favorece la separación de las nanoparticulas, previniendo el posible envenenamiento del catalizador evitando interacciones del Pd con compuestos de azufre (por ejemplo, grupos tiol presentes en las proteínas).

Hasta ahora, solo cultivos puros habían sido utilizados para la síntesis de nanopartículas, sin embargo, para aplicaciones prácticas esta condición tiene limitaciones. Por lo tanto, en este trabajo también se evaluó la capacidad de lodo granular metanogénico para reducir Pd(II) a Pd(0) mediante la adición de diferentes donadores de electrones. Los resultados obtenidos demostraron que es posible lograr la reducción de paladio y formación de nanopartículas empleando etanol como donador de electrones. Este estudió constituyó el primer reporte en el uso exitoso de un consorcio para la recuperación de Pd(II) y formación de nanopartículas de Pd(0) tanto en lote como en continuo empleando reactores tipo UASB (por sus siglas en inglés, upflow anaerobic sludge blanket). Además, la biomasa enriquecida con el Pd(0) fue utilizada en la transformación de contaminantes recalcitrantes de interés ambiental y se promovieron reacciones de

deshalogenación, hidrogenación y nitro-reducción como efecto del catalizador de paladio.

En este estudio se demostró que la biotranformación de dos compuestos modelo, iopromida (IOP) y 3-cloronitrobenceno (3-CNB) fue mejorada por la adición de Pd (como biomasa enriquecida con Pd(0)), esto pudo ser comprobado por estructuras simples formadas como resultado de la biotransformación del compuesto inicial. Los resultados obtenidos permitieron sugerir la ruta de biotransformación del 3-CNB, éste compuesto fue transformado, al final del tiempo de incubación, en dos productos principales, benceno y 1-amino-2-ciclohexeno, demostrándose que se promueven reacciones de nitro-reducción y deshalogenación.

IOP, un medio de contraste tri-yodado, fue biotransformado en estructuras más sencillas (se detectaron seis productos de transformación), los resultados sugieren la aparición de una estructura con un sustituyente yodado indicando deshalogenación de dos de los tres átomos que constituyen a la molécula. Esto constituye un paso importante hacia la mineralización de estos compuestos en un paso posterior.

Conjuntamente, el efecto inhibitorio de Pd(II) sobre la comunidad microbiana en ensayos en lote y en reactores UASB por periodos de operación prolongados fue evaluado y reportado en este estudio, lo cual contribuye a un mejor entendimiento de los puntos críticos en el control del proceso.

Los resultados obtenidos en este estudio demostraron que la recuperación y reducción de Pd(II) de efluentes que contienen este metal, tales como lixiviados ácidos, lixiviados de desechos electrónicos y catalizadores agotados, puede alcanzarse empleando un consorcio microbiano en un proceso continuo. Esto ofrece

la posibilidad de aplicar esta tecnología a escala de manera efectiva. Este trabajo propone un proceso biotecnológico para la recuperación de Pd de efluentes de desecho que contengan este metal para su aplicación posterior como bio-catalizador en sistemas de tratamientos de aguas para la biotransformación de contaminantes recalcitrantes.

Abstract

Aurora M. Pat-Espadas. 2015**. "Microbial reduction of palladium: mechanisms, modeling and applications in wastewater treatment systems".** Doctoral Thesis, IPICYT, San Luis Potosí, Mexico

The metabolic potential of microorganisms to change the oxidation state of metals is widely known and constitutes a powerful tool to develop and implement novel bioremediation techniques. Nowadays, not just the recovery of metals but also the production of metallic nanoparticles is a major issue and biotechnological approaches have emerged as promising options to accomplish this.

Palladium (Pd) is considered a precious metal with remarkable properties which make it attractive to many areas in industry. However, its abundance is limited on earth and efforts for its recovery when present in effluents and residues is limited. This dissertation focuses on this problematic by proposing a biological method for the recovery of Pd and the production of Pd(0) nanoparticles. With this objective, pure cultures of *Geobacter sulfurreducens,* strain PCA, and methanogenic granular sludge were evaluated to understand the mechanisms involved.

G. sulfurreducens is a microorganism with exceptional metabolic capacity, ubiquitous in sediments and soil, its hallmark physiological capability is of great interest from the biotechnological point of view. Results obtained demonstrated, for the first time, that *G. sulfurreducens* can couple the oxidation of acetate to the reduction of Pd(II) with the concomitant production of Pd(0) nanoparticles. Parameters such as cells:Pd ratio, medium composition, pH, speciation, Pd(II) concentration and the presence of a redox mediator were identified of significant

relevance in the process. Extracellular synthesis of Pd(0) nanoparticles was promoted using anthraquinone-2,6-disulfonate (AQDS) as redox mediator, which represents the advantage of a simple separation method and prevention of catalyst poisoning by avoiding Pd interactions with sulfurous compounds (e.g. thiol groups of proteins from bacteria).

Until now, only pure cultures of bacteria had been used for palladium nanoparticles production; however, for practical applications this condition would face limitations. Hence, in this work methanogenic granular sludge was studied for its ability to reduce Pd(II) to Pd(0) by using different electron donating substrates. Obtained results demonstrated that it is possible to achieve palladium reduction and nanoparticles production by providing ethanol as electron donor. For the best of our knowledge this study constitute the first report on the successful use of consortia, in batch and continuous mode in UASB (upflow anaerobic sludge blanket) reactors, to recover Pd(II) as Pd(0) nanoparticles. Furthermore, biomass enriched with biogenic-Pd(0) was proven to function as catalyst and promote reactions of dehalogenation, hydrogenation and nitro-reduction which are of special interest in the transformation of recalcitrant pollutants of environmental concern. In this study the biotransformation of two model compounds, 3-chloronitrobenzene (3-CNB) and iopromide (IOP), was demonstrated to be enhanced by the presence of Pd (using enriched-Pd(0) biomass) evidenced by the transformation of the parent compound into simpler structures.

Elucidation of 3-CNB biotransformation pathway showed that 3-CNB was transformed, at the end of the incubation time, into two main products, benzene and

1-amine-2-cyclohexene, demonstrated that nitro-reduction and dehalogenation occur.

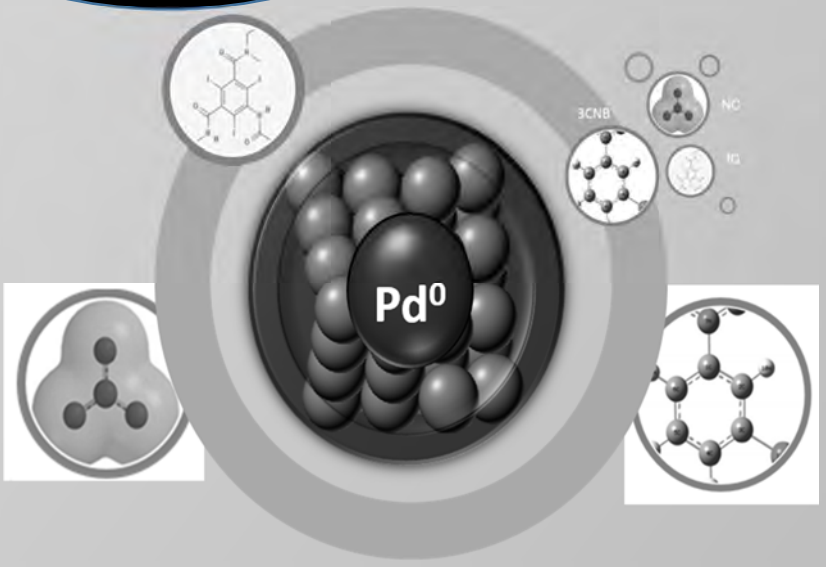
IOP, a tri-iodinated contrast medium, was biotransformed into simpler structures (six transformation products were detected); indeed results suggest dehalogenation to give one structure with one iodine which means the release of two of the three iodines constitutents of the molecule. This constitutes an important step towards their mineralization.

In addition, inhibitory effects of Pd(II) on the microbial community were evaluated in batch assays and in long-term UASB reactors operation, contributing to a better understanding of the critical points in process control.

The obtained results in this study demonstrated that the recovery and reduction of Pd(II) from waste streams, such as acidic leachates, electronic scrap leachates and spent catalyst which can serve as a secondary raw materials, can be achieved by a consortium in continuous process which open the possibility of applying this technology at full scale as an effective option. The present work proposes a biotechnological process to recover Pd from waste streams for its subsequent applications as bio-catalyst in wastewater treatment systems for the biotransformation of recalcitrant pollutants.

CHAPTER 1

INTRODUCTION



1. Introduction

Since many years ago it is well known that microbial metabolism can change the oxidation state of inorganic species, for instance by oxidation and reduction of metals.[1] Moreover, bacteria have shown extraordinary resistance to several toxic metal ions, which was proved by the identification of genes on bacterial plasmids.[2]

Cells carry out these processes by means of two uptake systems: (i) an unspecific type characterized by occurring fast and driven by chemiosmotic gradient across the cytoplasmic membrane of bacteria and (ii) a high substrate specificity system, slower process that uses ATP hydrolysis as the energy source, generally an induced system which occurs in cells in time of need, starvation or special metabolic situations.[3]

Bacteria and *Archaea* can use these systems to conserve energy for growth or respiration using metals as terminal electron acceptors under anaerobic conditions in a dissimilatory process.[4]

This exceptional capacity of microorganisms has led to the pursuit of biotechnological applications in order to integrate bioprocesses with new technologies developed from other areas of science and technology.[2]

1.1 Metallic nanoparticles production by microorganisms

Metals are ubiquitous on earth; however, as they are extensively used, for instance in mining and manufacturing, they have become an important environmental issue because of their non-degradable nature.[5] Moreover, high demand of metals has led to a decrease in reserves, reason for what metal recovery is of great relevance. Conventional recovery techniques namely pyrometallurgy and physicochemical techniques including precipitation, solvent extraction, etc., have some drawbacks such as lack of specificity, high energy and capital inputs, generation of secondary waste streams and poor effectiveness at low concentrations.[6,7] Hence, biotechnological approaches have emerged as feasible and environmentally friendly processes for metal recovery and production of metal nanoparticles that are of aggregate value.

Nanoparticles are of great interest because they exhibit unique electronic, magnetic, catalytic and optical properties which are different from those of bulk metals. For that reason nanoparticles are used in different fields and nowadays nanotechnological products have already reached the market of more than one thousand products including cosmetics, textiles, housing and construction, sport items, among others.[8] Chemical and physical methods are used to synthesize nanoparticles but they imply the use of toxic chemicals [9,10] reason for what biological methods represent an attractive option towards the development of green processes for nanoparticles production.

The use of microorganisms for remediation purposes has been applied since years ago; however, the approach of production of nanoparticles is very recent. Until now, there are several studies reporting on the production of nanoparticles by species of gram-negative and positive bacteria, magnetotactic bacteria, diatoms, S-layer bacteria, fungi, actinomycetes, yeast and leaf extracts.[11] Among them, bacteria have been widely explored for the synthesis of metal nanoparticles, including silver, gold, copper and cobalt.[12] Moreover, metal nanoparticles, such as platinum, zinc, titanium, palladium, iron, and copper have gained attention in recent times because of their technological importance.[13]

The mechanism of nanoparticles biosynthesis is generally described based on the ability of bacteria to reduce metals and to accumulate them in their elemental form through enzymes produced by cellular activity, but so far it has not been described a detailed mechanism.[11]

Since nanoparticles can be formed inside (by transporting ions into the microbial cell) or outside the cell (by trapping the metal ions on the surface), synthesis referred to as intracellular or extracellular, respectively. Figure 1.1 shows in these processes in schematic way.

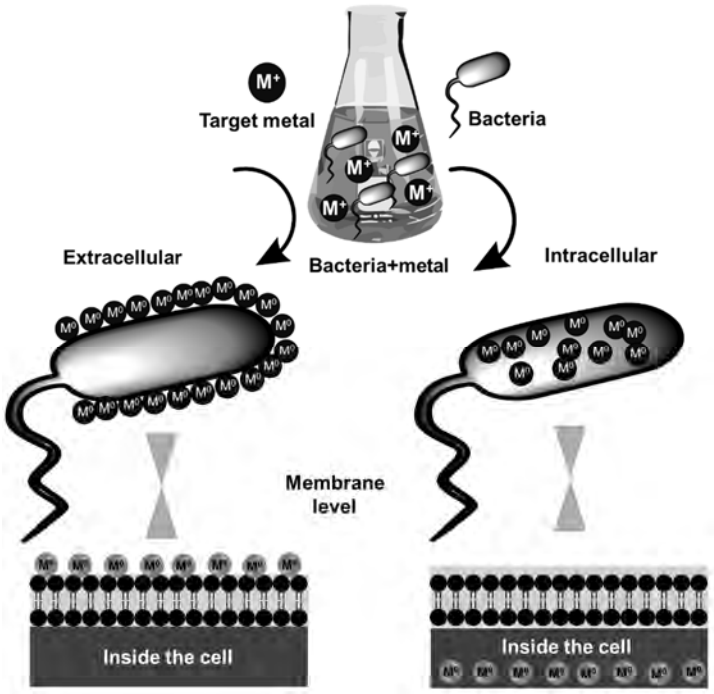


Figure 1.1 Intracellular and extracellular biological synthesis of nanoparticles

There are several reports regarding either intra- or extracellular synthesis of nanoparticles using different metal-reducing bacteria. Table 1.1 summarizes some representative studies reported in the literature. Up to now, there are more than 50 strains proven to synthesize different metal nanoparticles, mainly silver (Ag) and gold (Au).

Table 1.1 Different bacteria used to produce metallic nanoparticles

	Bacteria	Metal nanoparticle	Size range (nm)	Reference
Intracellular	*Pseudomonas stutzeri*	Ag	≈200	14
	Escherichia coli	CdS	2-5	15
	Escherichia coli DH5α	Au	25-33	16
	Shewanella algae	Au	10-20	17
		Pt	5	18
	Lactobacillus strains	Ag and Au	-	19
	Escherichia coli Recombinant	CdZn,CdSe,CdTe,SeZn	10.01,5.01	20
	Bacillus cereus	Ag	4-5	21
		ZnO	20-30, 25	22
	Enterobacter	Hg	2-5	23
	Rhodococcus sp.	Au	5-15	24
	Morganella sp.	Ag	20-30	25
	Klebsiella pneumonia	Ag	5-32	26
Extracellular	*Actinobacter*	Fe_3O_4	10-40, 50-150	27
		γ-Fe_2O_3	50	28
		Fe_3S_4	19	28
	Thermoanaerobacter ethanolicus	Fe_3O_4	>35	29
	Shewanella sp.	Fe_3O_4	26-38	30
	Shewanella loiha	Fe_3O_4	20-40	29

Table 1.1 (continued)

	Bacteria	Metal nanoparticle	Size range (nm)	Reference
Extracellular	*Geobacter sulfurreducens*	Ag	30	31
	Pseudomonas aeruginosa	Au	15-30	32
	Shewanella oneidensis	U (IV)	-	33
		Pd	-	34
		UO2	1-5	33
		Ag	4 ± 1.5	35
		Au	12 ± 5	36
	Desulfovibrio desulfuricans	Pd	50	37
	Lactobacillus sp. from yoghurt	Ag	15-25	38
	Lactobacillus sp. from probiotic tables	Ag	10-15	38
		TiO_2	10-25	38
	Bacillus subtilis	Ag	6.1±1.6	39
	Thermomonospora sp.	Au	7-12	40

1.2 *Geobacter sulfurreducens*: metabolic potential for practical applications

Geobacter sulfurreducens, a gram-negative δ-proteobacterium, is a dissimilatory metal- and sulfur-reducing microorganism isolated from surface sediments of hydrocarbon-contaminated ditch in Norman, Oklahoma by Caccavo et al.,.[41] The complete genome of *G. sulfurreducens* was sequenced in 2003, consisting in a single circular chromosome of 3,814,139 base pairs (bp) with a total of 3466 predicted protein encoding open reading frames [coding sequences (CDSs)].[42] This information facilitated the study and elucidation of different metabolic processes under different conditions. *G. sulfurreducens* has an impressive potential due to its metabolic and physiological versatility, which makes it attractive for practical

applications. The most important features of *G. sulfurreducens* metabolism are described in the following paragraphs.

Geobacter sulfurreducens can couple the oxidation of acetate or hydrogen to the reduction of metals; for instance, Fe (III) (in the form of poorly crystalline iron oxide (PCIO), ferric citrate (Fe(III)-cit), and ferric pyrophosphate, (Fe(III)-P), Tc(VII), Co(III) and U(VI)[41], humic substances[43], fumarate, elemental sulphur and malate are also used as electron acceptors.[41] Moreover, *G. sulfurreducens* can tolerate concentrations of 10% of O_2 and use it as terminal electron acceptor, surviving oxidative stress and growing on oxygen.[44] It also has the ability to transfer electrons to electrodes producing high current densities with very high coulombic efficiencies.[45,46]

In general, the capacity of *G. sulfurreducens* to reduce metals, including toxic and radioactive, can be attributed to its ability to transfer electron extracellularly, exchanging electrons to redox active species as a nonspecifically process. The latter is achieved by means of an exceptional mechanism in which cytochromes and multicopper proteins, as well as its pili, that is electrically conductive,[47,48] are involved.[49]

G. sulfurreducens genome encodes 89 cytochromes in total; moreover, 85% of its cytochromes contain more than one heme motif – with 7.5 hemes per cytochrome on average.[50] The great number of c-type cytochromes may have the additional role of functioning as capacitors storing electrons which can be carried out in the periplasm and in the outer-surface.[51]

Geobacter species have the potential to be applied in bioremediation strategies, such as degradation of aromatic compounds linked to Fe(III) reduction,[52] reduction of soluble ions of metals to less soluble forms, reductive dechlorination improvement with electrodes, development of large-scale microbial fuel cell systems for wastewater treatment, etc.[49]

1.3 Microbial consortia as an alternative to produce nanoparticles

Until now, pure cultures of bacteria have been used to produce nanoparticles. Nevertheless the use of consortia would imply advantages over pure cultures, for

instance granular biomass provides better biomass retention, tolerance to toxic substrates and ability to withstand shock loadings.[53–55]

Since most efforts have been focused on pure strains there is an opportunity regarding the use of consortia to produce metal nanoparticles which is worthy of study.

In fact, there are few and recent studies in relation to the use of consortia in batch conditions. For instance, granular aerobic mixed culture under fermentative conditions was tested in a very recent study.[55] Sulfate reducing bacteria (SRB) enrichment has also been proved to be able to reduce Pd(II) to Pd(0) in batch experiments.[56]

Anaerobic granular sludge would have a great potential towards metal reduction whenever the appropriate conditions are applied. Since granular sludge involves a wide variety of microbial species for instance, fermentative bacteria, hydrogen generating bacteria, homoacetogens, methanogen, etc., this could provide adequate conditions to promote metal reduction.

1.4 Palladium: a valuable metal

Palladium belongs to the group of precious metals (along with platinum, ruthenium, rhodium, osmium, iridium, gold and silver) and it is widely used in chemical catalysis, electrical appliances and jewelry, but the main reason of the increase in Pd demand is due to its use as automotive emission control catalyst.[57] Table 1.2 summarizes the main characteristics of palladium element which is a valuable metal with unique properties such as stability, high reactivity, and/or less toxic compounds when using it as catalyst.[58,59] A main aspect of this element regards its coordination characteristics based on Pearson's classification according to which palladium belong to class B and it is a 'soft' element preferentially coordinating with ligands of decreasing electronegativity[60].

Table 1.2 Technical data for palladium (Pd).[59]

Atomic number	46
Atomic weight	106.42
Discovery	1803, United Kingdom
World largest producers	Russia (44%) and South Africa (40%)
Block, group and period	d, 10, 5
Color	Silver
Oxidation state	+2, +4
Electronegativity	2.2
Density (20°C)/g cm^{-3}	11.99
Metal radius (12-coordinate)/pm	137
Magnetic type	Paramagnetic
Abundance % in Earth's crust	6.3×10^{-7}
Atomic radius/pm	169
Crystal structure	Face centered cube
Ionization energies/ kJ mol^{-1}	804.4, 1870, 3177
Melting point/°C	1554.9
Boiling point/°C	2963

Palladium can catalyze a wide range of reactions, for instance hydrogenation, hydrogenolysis, hydrodeoxigenation, hydrodehalogenation, carbonylation, formation of C–C, C–O, C–N, and C–S bonds, cycloisomerization, and even pericyclic reactions.[58,61] This property is mainly due to its great affinity to dihydrogen (H_2) since Pd can absorb up to 935 time its own volume of H_2.[59]

The increasing industrial use of this metal has led to its limited availability [62] causing rise and variations in price, situation that has encouraged the searching of new efficient techniques for the recovery of palladium from waste streams.

Moreover, the latest trend is the use of palladium nanoparticles (NPs) instead of bulk material to achieve better performance and results with less amount of metal since NPs offers high surface-area-to-volume ratio.[63]

Traditional methods for nanoparticles synthesis include chemical ('bottom-up') and physical ('top-down'), with production processes generally involving an important number of chemicals which usually are toxic; moreover, these methods involve consumption of energy, factors that combined make NPs production very expensive[64].

For those reasons researching is being focused on biological methods that can offer an environmentally friendly process towards a sustainable production of nanoparticles as well as a feasible option for their recovery from industrial wastes.

1.5 Palladium in the environment and their potential risk

Palladium is present in nature in low concentration in earth crust (mine) but its occurrence in the environment is because of anthropogenic emission related to the use of this element, mainly in catalytic converters for automobile exhaust purification, together with platinum (Pt) and rhodium (Rh). Typically an automobile catalytic converter contains 0.08% Pt, 0.04% Pd and 0.005-0.007% Rh supported on a base.[65]

Catalytic converters are employed in the treatment of pollutants in exhaust gases from motor vehicles (Figure 1.2), their function is to reduce the emission of toxic pollutants, for instance, carbon monoxide (CO), unburnt hydrocarbons and nitrogen oxides (NOx).[66] The catalyst promotes reactions of oxidation/reduction to transform the contaminants into carbon dioxide (CO_2), water (H_2O) and nitrogen (N_2) and this technology has been used for over 20 years.[67,68]

The intensive use and application of platinum group elements (PGE), in general, has resulted in some undesirable effects, such as widespread distribution of fine particulate matter, or dust, containing PGEs, which originates from abrasion and deterioration of the bulk catalysts, as well as from mechanical and thermal impact on the active layer of automobile catalysts.[67,69]

Moreover, according to a study by Gaita and Al-Bazi[70], each year approximately 10 million automobiles are scrapped only in the United States, which is equivalent to estimate that 155 ton of Pt, 62 ton of Pd and 16 ton of Rh are annually wasted.

These elements may enter the environment by binding (either complex-ionic, ∏-complexes or chelate-binding)[71] with complexing materials present in nature such as humic substances.[72] Hence, contamination with PGEs can occur in airborne particulate matter (PM), roadside dust, soil, sludge and water, etc., which would lead to bioaccumulation of these elements inx| living organisms through diverse pathways.[73]

As a result different investigations focused on quantifying these elements in geological, industrial, biological and environmental samples as follows.[66]

Blood and urine. Results of analysis of palladium concentrations in blood and urine of nonexposed personnel reached levels of 32-78 ng L^{-1} and ≈10 ng L^{-1}, respectively.

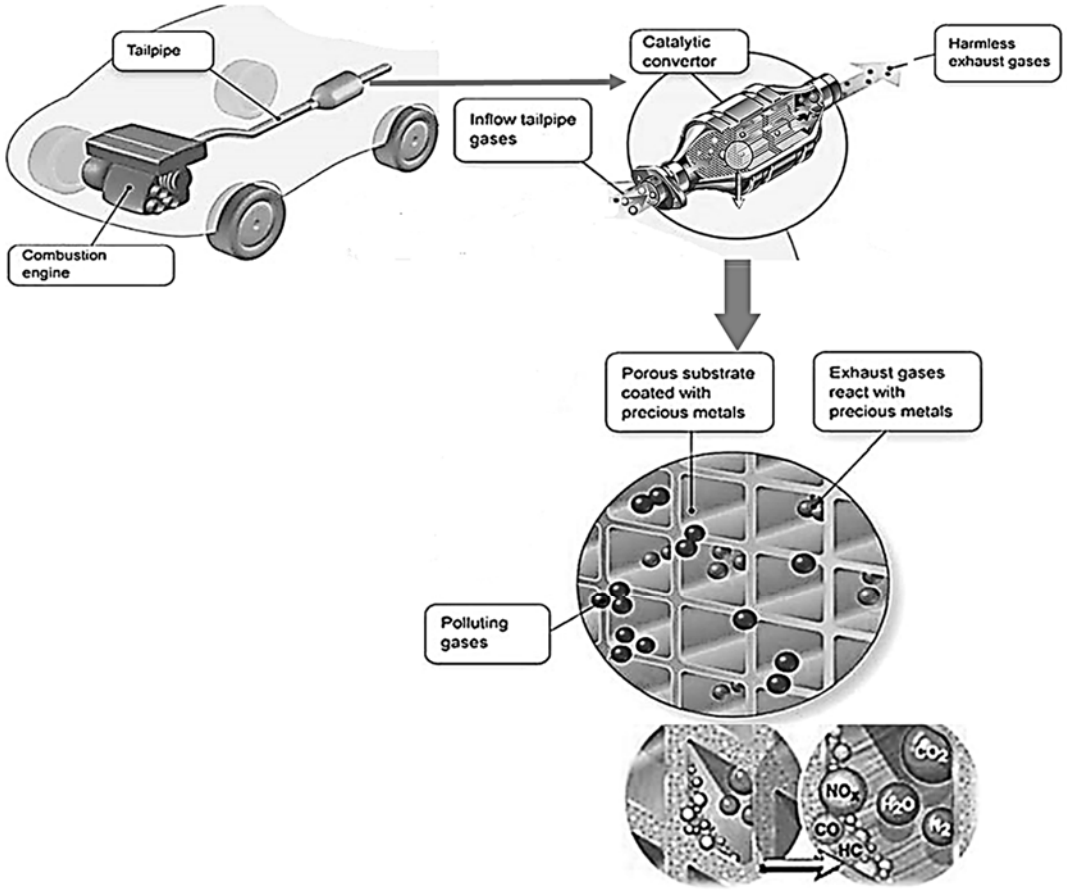


Figure 1.2 Scheme of auto catalytic converter (Modified from reference 68)

Airborne. Determinations of PGE concentration in airbone PM is limited by analytical methods because there are many aspects interfering the measurement. Table 1.3 shows an overview on the PGE content of airborne samples.

Soil, dust and plants. In these matrixes, analysis of samples has revealed that the concentration of Pd (and PGEs in general) exceeds the natural background level. (Table 1.4). Indeed, Schäfer et al.,[74] demonstrated that the order of plants uptake rates is: Pd>Pt>Rh with an uptake of the noble metal into the different organs of the plants in the decreasing order: root>stem>leaf.[74]

Table 1.3 Pd concentration in airborne samples.[73]

Place	Sampling observation	Pd concentration (pg m^{-3})	Reference
California, USA		<0.06	75
	PM_{10}	1	76
Chicago, USA		12 700	77
Rome, Italy	Urban sites(heavy traffic)	21.2–85.7	78
Milan, Italy		<0.7	79
Caesarea, Israel	$PM_{2.5}$	3.3	80
Chernivtsi, Ukraine		56 600	77
Czech Republic	Various observation sites	30-280	81
Germany	Not specified	9-106	82
Berlin	Urban	0.2-14.6	83
Madrid, Spain	M-30 highway (PM_{10})	5.1-32	84
	Weekly average (PM_{10})	2.6	
Vienna, Austria	Weekly average (for particle size <30mm)	14.4	85

Natural water (rivers, coastal and oceans). The presence of Pd in these matrixes is linked to the released metal from vehicle exhaust catalysts and its mobility, which could be related to its chemical form and its interaction with organic matter. In addition, electroplating waste associated with industries can release dissolved or readily dissolvable compounds of PGEs.[86]

Table 1.4 Pd concentration in soil and road dust samples.[73]

Place	Sampling observation	Pd concentration (ng g^{-1})	Reference
Mexico City	Insurgentes/Periférico (soil) (200 cars min^{-1} at rush hours)	53.2-74	87
	Insurgentes/Sta. Teresa (60 cars min^{-1} at rush hours)	12.2-32.4	
	Periférico (110 cars min^{-1} at rush hours)	15.2-82.7	
	Periférico/Viaducto (180 cars min^{-1} at rush hours)	62.5-101.2	
San Diego, Cal,	Roadside dust heavy traffic	38–280	88
Germany	Roadside dust Highway	1-146	74
	Frankfurt	6-117	89
Japan	Tunnel dust	297	69
Sweden	Road dust	56	90
Madrid	Road dust ring road, city center	39-191	91

Palladium presence, in electrical waste and electronic equipment scrap leachate (WEEE), waste printed circuit boards, etc., constitutes an important source of release of this metal (an others) to the environment.

For example, composition of printed circuit board scrap is up to ≈30 mg L^{-1} of Pd, among other elements such as Au (60-113 mg L^{-1}), Cu (20-25wt%)[92]. Moreover, a typical leachate of a manufacturing company encompassing refining and management of precious metals, catalysts and other precious metal products, electro-optic and electronic materials contains a mixture of Ag (1 mg L^{-1}), Au (9 mg L^{-1}), Pt (280 mg L^{-1}), Pd (110 mg L^{-1}), Rh (93 mg L^{-1}), Ir (2 mg L^{-1}), Ru (2 mg L^{-1}), Pb (29 mg L^{-1}), Cu (5 mg L^{-1}) and traces of Bi, Te, Se, Fe, and Sb with highly acidic pH around 1.4.[93]

To give an idea of the high content of valuable metals contained in waste: (i) one ton of mobile phones (approximately 6,000 handsets) contains about 3.5 kg of silver, 340 g of gold, 140 g of palladium and 130 kg of copper;[94] (ii) a personal computer contains 0.081 g of Pd, 5570 g of Fe, 1880 g of Cu, 3850 g of Al, 5.13 g of Ag, among others.

These numbers evidenced the problematic of metal waste generation and the urgency of recover them using an eco-friendly process to contribute to sustainable resource management.

1.6 Relevance of palladium recovery

The process proposed, as result of this investigation, is of relevance since palladium is a scarce metal on earth (average concentration in lithosphere of 0.015 mg kg^{-1})[73] and its recovery has become an important issue. Palladium is extensively used in the catalytic control of car exhaust emissions combined with Pt. In the year 2000, the worldwide demand of palladium by application was around 61.3% of Pd in autocatalysis, 24.6% in manufacture of electronic components, 10.4% in dentistry and 6.3% for other purposes[73], and in that year the worldwide demand of Pd was 238 Ton. By 2012, statistics reported a mine production of 200 Ton, Russia and South Africa lead the production of this PGM with 41% and 36% of mine production, respectively, as it can be seen in Figure 1.3, but the largest reserves are in the

Bushveld Complex in South Africa. World resources of PGMs in mineral concentrations that can be mined economically are estimated to total more than 100 million kilograms.[95] Hence, the supply and demand of palladium is variable which impact the price; indeed, in the last years its price fluctuated around 16 € g^{-1} and 22 € g^{-1}.[96] Moreover, the elimination (or recovery) of PGMs from metal-containing waste is s an imperative necessity because their inertness is considered harmless for a long period of time. This scenario has encouraged the recovery of this metal by using promising techniques such as biological methods.

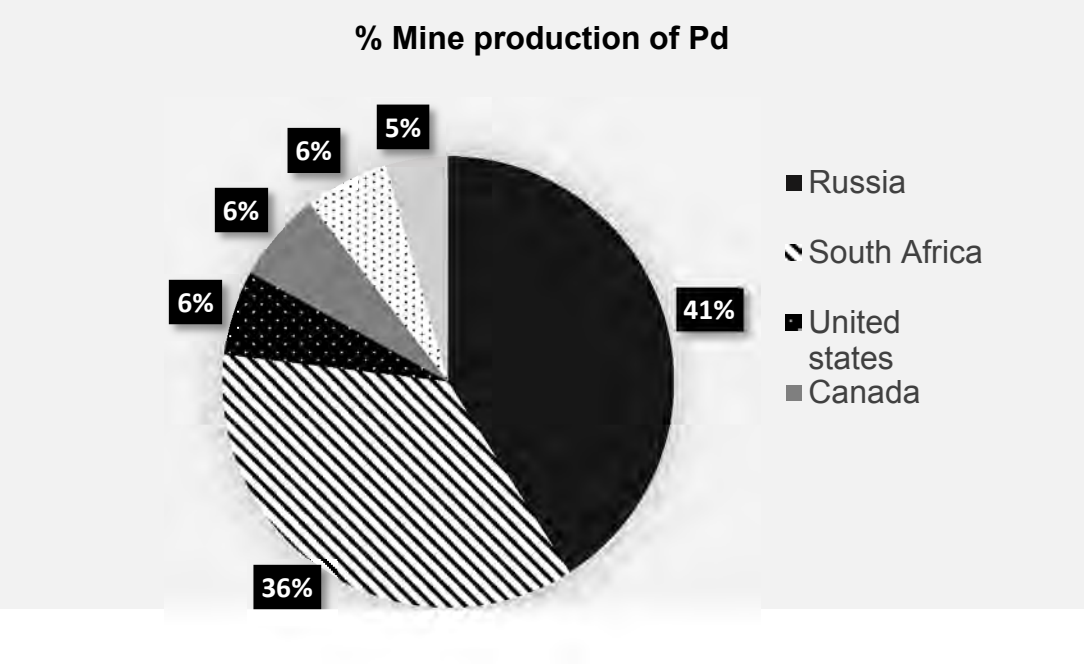


Figure 1.3 Worldwide mine production of palladium. Values according the Mineral commodity summaries, 2013.

1.7 Palladium recovery and nanocatalyst production by microorganisms: applications

Because of the great demand of palladium and particularly in the form of nanoparticles, biological methods of production have been raised as a promising alternative with an environmental friendly approach.

Furthermore, biological production of Pd nanoparticles is now positioned as an innovative method that allows both metal recovery and nanocatalyst synthesis.

Microbially produced Pd(0) can be applied in a wide range of biotechnological or industrial areas, for instance it was successfully applied to remove a wide range of environmental contaminants. Moreover it has been proved the superior catalytic activity of biological Pd nanoparticles as compared to commercial Pd(0) powder.[97,98] Table 1.5 shows an overview of different biotechnological applications of Pd(0) regarding degradation of environmental pollutants.

1.8 Scope and outline of thesis

Precious metals are scarce on earth and extensively used for industrial purposes situation that generates two scenarios: (i) urgency of recovery to satisfy demand and (ii) environmental pollution control.

Biological mediated reduction of metals is a promising alternative to solve both problems regarding the presence of metals in the environment. For practical applications, the process needs to be fully understood which involves mechanisms, process parameters of influence, limiting factors, etc. Moreover it is necessary to develop a sustainable and practical process to offer a realistic alternative to the production of metal nanoparticles and metal recovery.

For that reason, the aim of this dissertation was to evaluate the ability of microorganisms to reduce Pd(II) to Pd(0) and the further use of this biogenic-Pd(0) as catalyst in the biotransformation of model priority water contaminants. For these reasons, the investigation was divided in three parts: (i) evaluation of a pure strain to reduce Pd(II) and study the mechanisms involved, (ii) evaluation of methanogenic consortia to reduce Pd(II) for its immobilization as Pd(0) in anaerobic granular sludge and (iii) application of the biogenic-Pd(0) in the transformation of two model priority contaminants.

Table 1.5. Overview of different catalyzed reactions by biogenic Pd(0) nanoparticles.[99]

Compound	Polluted site/type of reaction catalyzed	Pd-reducing strain	Reference
Cr (VI)	Industrial wastewater reduction	*Desulfovibrio desulfuricans*	100
		Desulfovibrio vulgaris	101
		Escherichia coli	100
		Clostridium pasteurianum	102
ClO_4^-	Groundwater and drinking water reduction	*Shewanella oneidensis*	103
Polychlorobifenyls (PCBs)	Air, water, soil, sediments dechlorination (1–10 Cl)	*Desulfovibrio desulfuricans*	104
		Desulfovibrio vulgaris	104
		Shewanella oneidensis	97
Chlorophenols	Dechlorination (1–10 Cl)	*Desulfovibrio desulfuricans*	104
		Desulfovibrio vulgaris	104
Lindane	Soil and groundwater dechlorination (6 Cl)	*Shewanella oneidensis*	98
Trichloroethylene (TCE)	Groundwater dechlorination (3 Cl)	*Shewanella oneidensis*	105
Polybrominated diphenyl ethers (PBDE)	Indoor air and dust debromination (1–10 Br)	*Desulfovibrio desulfuricans*	106,107
Iodinated contrast media (ICM)	Wastewaters and surface waters deiodination (3 I)	*Shewanella oneidensis*	108,109
		Citrobacter braakii	110

In Chapter 2, the capacity of *G. sulfurreducens* to use Pd(II) as terminal electron acceptor with the overall goal to produce Pd(0) NPs was evaluated, two main aspects were studied: the cells quantity and the influence of the quinone model compound, anthraquinone-2,6 disulfonate (AQDS). The mechanisms involved during the microbial reduction of Pd(II) to produce Pd(0) NP's by *Geobacter sulfurreducens* were elucidated and a mathematical model was developed. These results are described in Chapter 3.

In Chapter 4, methanogenic granular sludge was examined for its capacity to reduce Pd(II) to Pd(0), different electron donating substrates were tested and the inhibitory effect of Pd(II) on the acetoclastic and hydrogenotrophic methanogenic activity was reported. The production of biogenic Pd(0) in a continuous process using laboratory-scale UASB (upflow anaerobic granular sludge bed) reactors inoculated with methanogenic granular sludge was achieved and it is described in Chapter 5. The application of a mixture of anaerobic granular sludge and biogenic-Pd(0) was tested in the biotrasformation of iopromide and 3-chloronitrobenzene in continuous UASB reactors, which is described in Chapter 6. Finally, the results obtained in this research are globally discussed in Chapter 7.

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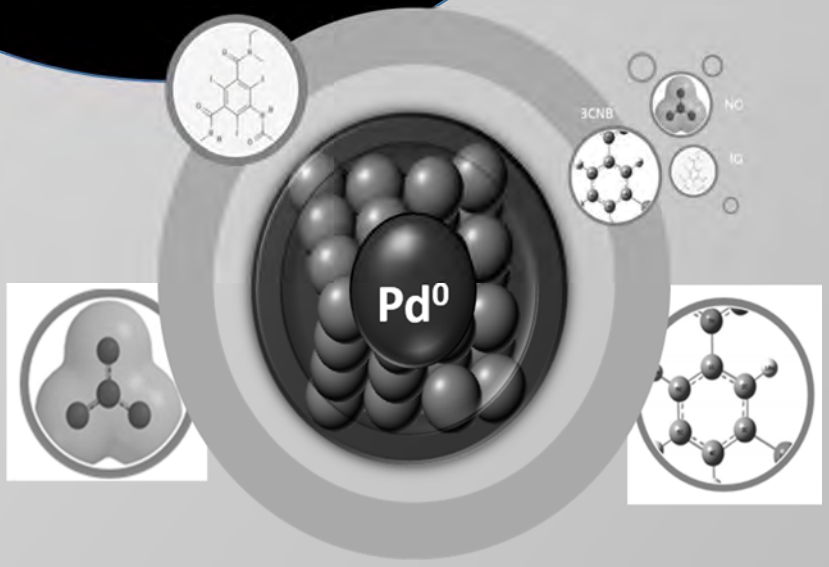
CHAPTER 2

Reduction of palladium and production of nanocatalyst by *Geobacter sulfurreducens*



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2.1 Abstract

This chapter describes for the first time the ability of *G. sulfurreducens* PCA to reduce Pd(II) and produce Pd(0) nano-catalyst, using acetate as electron donor at neutral pH (7.0±0.1) and 30ºC. The microbial production of Pd(0) nanoparticles (NPs) was greatly enhanced by the presence of the redox mediator, anthraquinone-2,6-disulfonate (AQDS) when compared with controls lacking AQDS and cell-free controls. A cell dry weight (CDW) concentration of 800 mg L^{-1} provided a larger surface area for Pd(0) NPs deposition than a CDW concentration of 400 mg L^{-1}. Sample analysis by transmission electron microscopy revealed the formation of extracellular Pd(0) NPs ranging from 5 to 15 nm and X-Ray diffraction confirmed the Pd(0) nature of the nano-catalyst produced. The present findings open the possibility for a new alternative to synthesize Pd(0) nano-catalyst and the potential application for microbial metal recovery from metal-containing waste streams.

2.2 Introduction

Development and advance in several nanotechnology fields have favored the exploration and application of nanometric materials in many areas. The exploitation of metallic nanoparticles (NPs) has grown as their importance became recognized.[1] This is the case of ultrafine transition metal NPs, which can be used in diverse fields, such as catalysis and disinfection,[2–4] electronics,[5] optics,[6] and even in biological and medical science.[7] Lately, the interest in palladium (Pd) NPs has grown because they can be used in both homogeneous and heterogeneous catalysis, which is due to their high surface area to volume ratio and to their high surface energy.[4] In the field of water treatment, Pd-based catalysts offers advantages because of their ability to activate di-hydrogen (H_2) and to catalyze reductive transformation of a number of priority contaminants (*e.g.* pollutants recognized for their adverse effects on the environment and on public health).[8,9]

Moreover, there is a growing interest in synthesizing metal NPs by biological methods since the processes occur at neutral pH and close to ambient temperature and pressures. Hence, these synthesis processes are considered less labor-

intensive, low-cost techniques which do not produce toxic byproducts and constitute a promising area of green chemistry.[3,10,11]

The main reasons to explore bacterial cultures to produce metal nano-catalysts are their variety, abundance in different environments, relative simplicity of their manipulation and their metabolic versatility, which allows the microbial reduction of a wide variety of metals that can serve as terminal electron acceptors to form nano-catalyst[12]. Previous studies have documented the capacity of different microorganisms to reduce Pd(II). *Desulfovibrio desulfuricans* and *Shewanella oneidensis* are considered the most studied organisms in the context of the bioreductive synthesis of Pd(0) NPs.[13–15] Pd(0) NPs formed at the outer surface of the bacterial cells[14,15] may be exploitable as catalysts in the transformation of contaminants, for instance. Specific examples are the application of Pd nano-catalyst in the reductive transformation of Cr(VI), ClO_4^-, polychlorobiphenyls, chlorophenols, trichloroethylene and iodinated contrast media.[13] Another environmentally beneficial application is the microbial production of Pd(0) NPs in the treatment of industrial effluents containing Pd, such as waste streams from spent automotive catalysts and printed circuit boards processes, which contain substantial amounts of Pd and other precious.[13]

G. sulfurreducens, a delta-proteobacterium, is known for its versatile ability to couple the oxidation of acetate or hydrogen to the reduction of Fe(III), Co(III), U(VI), Tc(VII), fumarate and humic acids.[16–20] Furthermore, *G. sulfurreducens* is able to produce electricity by using anodes as terminal electron acceptor in microbial fuel cells.[21]

Recently, several studies have documented the important contribution of humic substances and quinone analogues to the reduction of metals, such as Fe(III), Mn(IV), Tc(VII) and Cr(VI).[22,23] The addition of catalytic concentrations of humic substances or quinones to microbial cultures has significantly increased the rate of reduction of these metals. In some cases, the presence of humic substances or quinones as redox mediators is even a prerequisite to achieve the microbial reduction process. Moreover, it has recently been reported that different quinone redox mediators enhanced the production of Se(0) and Te(0) NPs by *Escherichia coli*.[24]

The aim of this work was to evaluate the capacity of *Geobacter sulfurreducens* to use Pd(II) as terminal electron acceptor with the overall goal to produce Pd(0) NPs during cultivation of this strain. The study further explored the catalytic effects of the quinone model compound, anthraquinone-2,6-disulfonate (AQDS), on the microbial production of Pd(0) NPs, which had not been previously reported.

2.3 Materials and methods

2.3.1 Microorganism and culture conditions

Geobacter sulfurreducens strain PCA (DSM 12127; ATCC51573) used in this study was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). The strain was routinely cultured anaerobically in acetate:fumarate medium as previously described[25,26] at 30°C.

2.3.2 Microbial reduction of Pd(II)

Late logarithmic phase cultures of *Geobacter sulfurreducens* were harvested by centrifugation and washed twice with a sterilized, osmotically balanced buffer (g L^{-1}): $NaHCO_3$ (2.5), NH_4Cl (0.25), $NaH_2PO_4 \cdot H_2O$ (0.006), KCl (0.1), NaCl (1.75).[27] Washed cells were re-suspended in glass serum bottles using 100 mL of a designed reaction buffer[27] containing (g L^{-1}): $NaHCO_3$ (2.5), NH_4Cl (0.25), $NaH_2PO_4 \cdot H_2O$ (0.006), KCl (0.1). Serum bottles were capped with inert viton stoppers and all anaerobic media were flushed with N_2/CO_2 (80%/20%). Headspace volume (22 mL) was also flushed with the same gas mixture.

Medium was inoculated with washed cells to yield one of two different final cell concentrations, 400 or 800 mg L^{-1} in terms of cell dry weight (CDW). The basal medium contained 5 mM acetate to serve as electron donor and Na_2PdCl_4 (Sigma-Aldrich) as sole terminal electron acceptor at a concentration of 25 mg Pd(II) per L. Depending on the experimental set-up, basal medium was supplemented with 100 µM of AQDS as redox mediator. Cell suspensions were dispensed into sterile glass serum bottles and incubated for 24 h at 30°C (100 mL of medium into glass serum bottles). All experimental treatments were set up in triplicate. The set-up was

sampled at specified time intervals in order to follow the reduction kinetics by analyzing Pd concentration and acetate consumption.

When hydrogen was supplied as electron donor, the headspace of the bottles was saturated with a mixture of H_2/CO_2 (80%/20%). Abiotic controls were incubated in cell-free media.

A diagram of Pd speciation in the medium at 0.5 pH intervals between pH 6 and 8 at 30ºC (total Pd concentration of 25 mg L^{-1} in the medium) was constructed using Visual MINTEQ® software ver. 3.0.

2.3.3 Analytical techniques

To document microbial reduction of Pd(II), 5 mL of appropriate samples of incubated inoculated or uninoculated Pd medium were filtered using 0.22 µm membrane filters (Millipore, Bedford, USA). Filtrate and filter deposit (Pd-associated with biomass or agglomerated) were analyzed separately by inductively coupled plasma-optic emission spectroscopy (ICP-OES, Varian 730-ES). The fraction retained on the filters was boiled with aqua regia, diluted and filtered with the same kind of membrane as before analysis.

Acetate consumption was analyzed by capillary electrophoresis (Agilent 1600A, Waldbronn, Germany). Acetate was quantified by comparison with high purity standard. A fused silica capillary column (Agilent, id 50 µm, 80.5 cm long, effective length 72 cm) and a basic anion buffer (Agilent, pH 12.1) were used. The temperature and voltage applied were 20 °C and −30 kV, respectively. Samples were injected with a pressure of 300 mbar for 6 s. Detection was carried out with indirect UV using a diode-array detector. The signal wavelength was set at 350 nm with a reference at 230 nm. A buffer flush for 4 min at 1 bar was performed prior to each run.

2.3.4 Transmission electron microscopy

Samples from different assays were prepared for transmission electron microscopy (TEM) by fixation in 3% glutaraldehyde for 2 h followed by three washing cycles (6,000 *g* for 10 min) with the buffer described above. Samples were collected on

copper grids (mesh size, 200 μm) covered with a carbon-coated Formvar film, and stained with 2% uranyl acetate. Preliminary observations were carried out with a JEOL electron microscope model 200 CX, operated under standard conditions at 80 kV in order to assess the best requirements for fine analysis of samples.

By using High resolution Transmission Electron Microscopy (HRTEM), electron-dense zones were located, where palladium could be deposited. In order to corroborate these data, energy-dispersive X-ray microanalysis (EDX) was used. Samples were examined by using a FEI field emission transmission electron microscope, model TECNAI F30.

2.3.5 X-ray diffraction analysis

Analysis of the palladium deposited on *G. sulfurreducens* cells was conducted in an X-Ray diffractometer Bruker D8 Advance. Samples were washed with chloroform-methanol (1:1, vol/vol) and then with acetone, and subsequently dried at room temperature as described previously by Lloyd et al. (1998).[15] X-ray diffraction (XRD) patterns were recorded from 20°-90° 2θ with a step time of 2 s and step size of 0.01° 2θ.

2.4 Results

2.4.1 Reduction of Pd(II) by *Geobacter sulfurreducens*

The speciation diagram (Figure 2.1) showed that $Pd(NH_3)_4^{2+}$ is the predominant species, representing 99.08% of the total palladium concentration (which correspond to 24.77 mgL^{-1} $Pd(NH_3)_4^{2+}$) followed by $Pd(NH_3)_3^{2+}$ representing 0.89% (corresponding to 0.22 mgL^{-1} $Pd(NH_3)_3^{2+}$) .

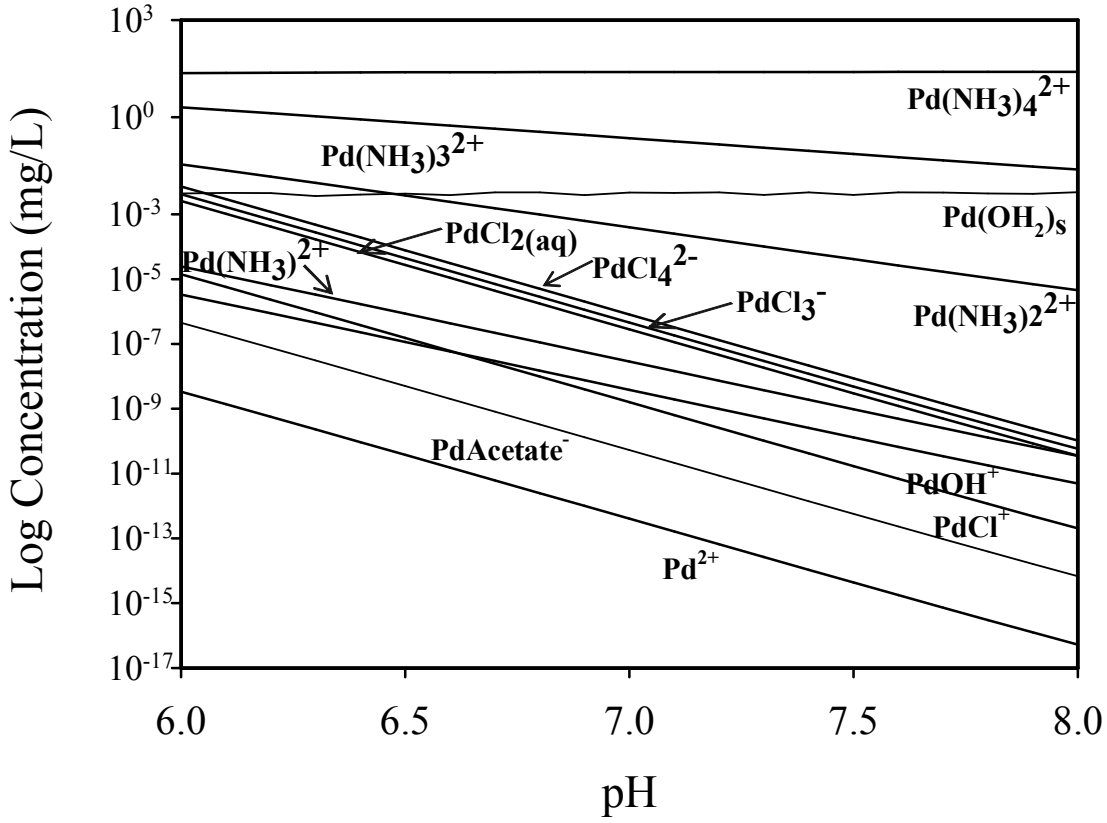

Figure 2.1 Speciation diagram of palladium in the reaction medium under the following experimental conditions: 25 mg Pd(II) L^{-1}, incubation temperature 30°C and pH 7. Diagram calculated using Visual MINTEQ® 3.0.

Other palladium species, such as $Pd(NH_3)_2^{2+}$, $PdCl_4^{2-}$, $PdCl_3^-$, $PdCl_{2(aq)}$, $Pd(NH_3)^{2+}$, $PdOH^+$, $PdCl^+$ and Pd^{2+} represented less than 0.001% (Table 2.1).

Table 2.1 Distribution of palladium species and standard reduction potentials (E°), at 298.15 K (25°C), and at a pressure of 101.325 kPa (1 atm) [a]

Species	Concentration (mg Pd L^{-1})	% of total concentration	E° (V)
$Pd(NH_3)_4^{+2}$	24.77	99.08	-0.024
$Pd(NH_3)_3^{+2}$	0.22	0.89	-0.214
$Pd(OH)_{2(s)}$	0.0047	0.02	+0.07
$Pd(NH_3)_2^{+2}$	NS	NS	+0.101
$PdCl_4^{2-}$	NS	NS	+0.62
$PdCl_3^-$	NS	NS	+0.567
$PdCl_2$ (aq)	NS	NS	+0.638
$Pd(NH_3)^{+2}$	NS	NS	+0.457
$PdOH^+$	NS	NS	+0.983
$PdCl^+$	NS	NS	+0.771
Pd^{2+}	NS	NS	+0.915

[a]Values referred to hydrogen standard electrode and were obtained from Bard et al. (1985), Bratsch (1989), Milazzo et al. (1978), Meng and Han (1996).[28–31] NS, Values considered as not significant since they represent less than 0.0001% of total palladium concentration.

From these results, it was assumed that under the experimental conditions applied, no significant formation of the precipitate $Pd(OH)_{2(s)}$ was produced, since this solid represented just 0.02% of the total concentration of Pd (0.0047 mg L^{-1} of Pd_{total}). Therefore, considering these negligible interferences, results derived from microbial incubations demonstrated that *G. sulfurreducens* readily reduced Pd(II) to Pd(0) linked to the anaerobic oxidation of acetate. The microbial reduction of Pd(II) mainly occurred during the first 2 h of incubation reaching a plateau after this period of time when the inoculum was 800 mgL^{-1} (CDW) (Figure 2.2A).

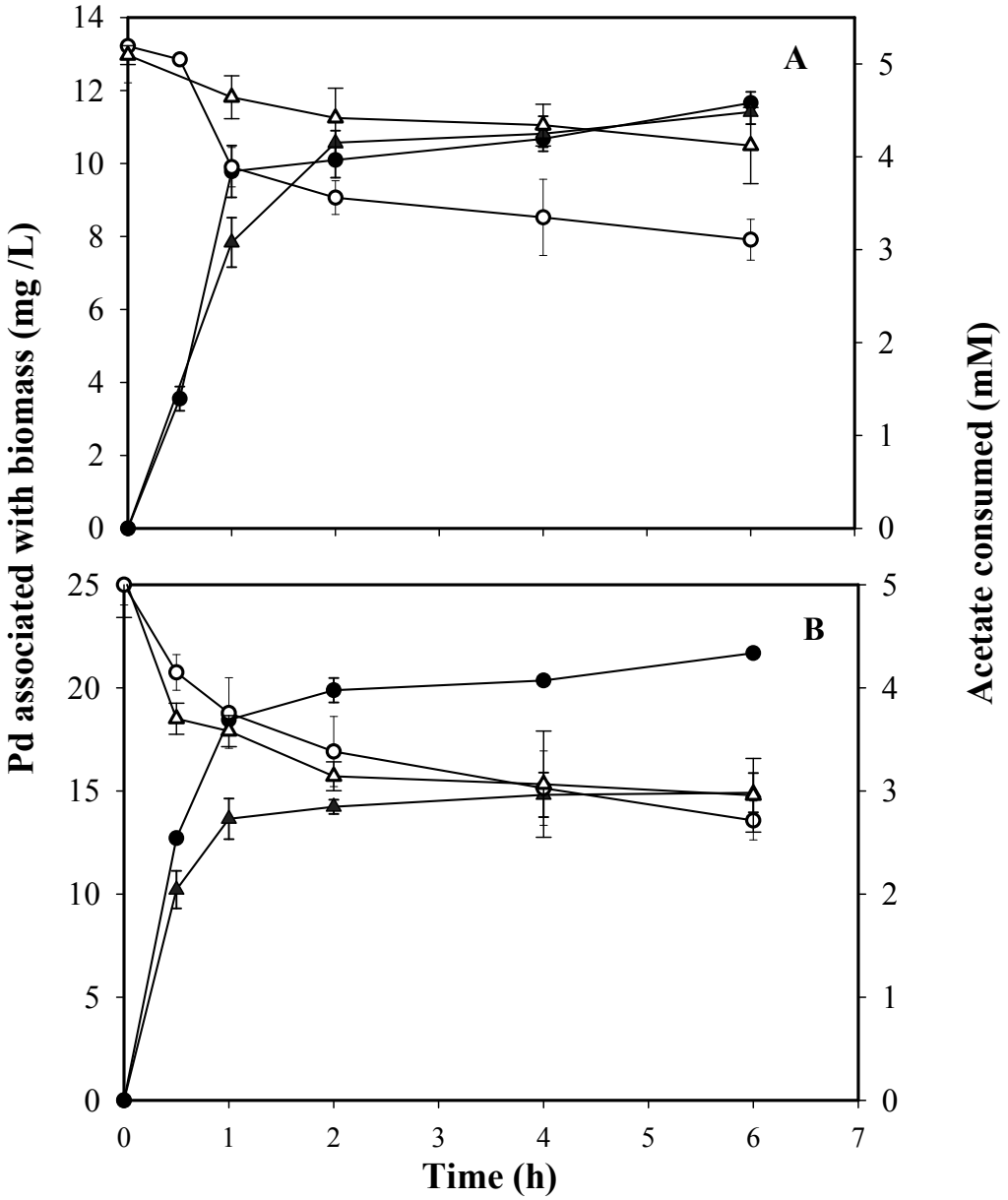

Figure 2.2 Kinetic evolution of Pd reduction and acetate consumption for (A) CDW 400 mgL^{-1} and (B) CDW 800 mgL^{-1}. Pd associated with biomass *G. sulfurreducens* Acetate consumed *G. sulfurreducens* Pd associated with biomass *G. sulfurreducens* + AQDS Acetate consumed *G. sulfurreducens* + AQDS.

Table 2.2 Reduction of Pd(II) by *G. sulfurreducens* after 24 h of incubation under different conditions. Incubations were performed in basal medium containing acetate as electron donor (5 mM). Treatments including AQDS were supplied with 100 µM AQDS.

Experiment [a]	Specific reducion rate (mg Pd g^{-1} CDW h^{-1})	Final Pd concentration in solution (mg L^{-1})	% Pd associated with biomass	% Pd remaining in solution	Recovery balance (%)[b]
G. sulfurreducens 800	17.06	3.52±0.77	83.01±2.34	16.99±1.15	99.91±0.09
G. sulfurreducens 800+AQDS	23.05	0.39±0.23	97.62±0.95	1.68±0.83	99.30±0.70
G. sulfurreducens 400	6.64	9.57±0.57	56.35±2.56	41.97±2.16	98.32±1.68
G. sulfurreducens 400+AQDS	13.25	1.02±0.71	89.01±2.13	4.44±1.22	93.41±6.59

[a]Value indicates the cell dry weight concentration (in mg L^{-1}) used. In the case of Pd, its distribution is reported as concentration of Pd remaining in solution; the percentage recovered from total Pd(II) added (25 mg L^{-1}), in the fraction associated with biomass and the percentage remaining in solution. All the experiments were done in triplicate. The results are presented as mean calculated value ± standard deviation.

[b]Recovery balance was calculated as the percentage obtained from [final Pd in solution + Pd associated with biomass]/ initial Pd *100.

According to the results summarized in Table 2.2, for the experiments conducted with 800 mgL^{-1} of CDW, palladium reduction was 2.6 times faster than with an inoculum of 400 mgL^{-1} (CDW) (Fig. 2.2B). Increase in biomass, in 24 h of incubation, also resulted in more extensive palladium deposition on the biomass (Table 2.2). Addition of AQDS as redox mediator further enhanced the microbial reduction of Pd(II) by *G. sulfurreducens* (Table 2.2). Indeed, the specific reduction rate of Pd(II) increased 1.35− and 2−fold by the supply of this redox mediator in microbial incubations performed with 800 and 400 mgL^{-1} of CDW, respectively, as compared to controls lacking added AQDS. Neither acetate oxidation nor reduction of Pd(II) was detected in uninoculated controls with or without added AQDS (data not shown). Moreover, the pH remained constant (7.1±0.1.) during the microbial reduction of Pd(II) by *G. sulfurreducens* in all experimental treatments. The reduction of Pd(II) was concomitant with an evident color change of the biomass which turned black.
Further experiments were performed to evaluate the capacity of *G. sulfurreducens* to reduce Pd(II) with H_2 as electron donor (data not shown). Attempts to distinguish between the microbial and chemical reduction of Pd(II) did not clearly establish a reduction attributable to *G. sulfurreducens* activity under these conditions. In both microbial and abiotic incubations complete reduction of Pd(II) occurred within 1 h (data not shown).

2.4.2 Characterization of microbially produced Pd nano-catalyst

Palladium-covered cells were observed and analyzed by means of TEM and HRTEM. From Figure 3A it can be seen that palladium reduction by *G. sulfurreducens* resulted in the deposition of Pd NPs ranging from 5 to -15 nm on the cell surface. Moreover, TEM images obtained from AQDS-amended cultures revealed that produced Pd NPs were not only associated to *G. sulfurreducens,* but also formed extracellularly at an average distance of up to 300 nm from the cell surface (Figure 2.3B).

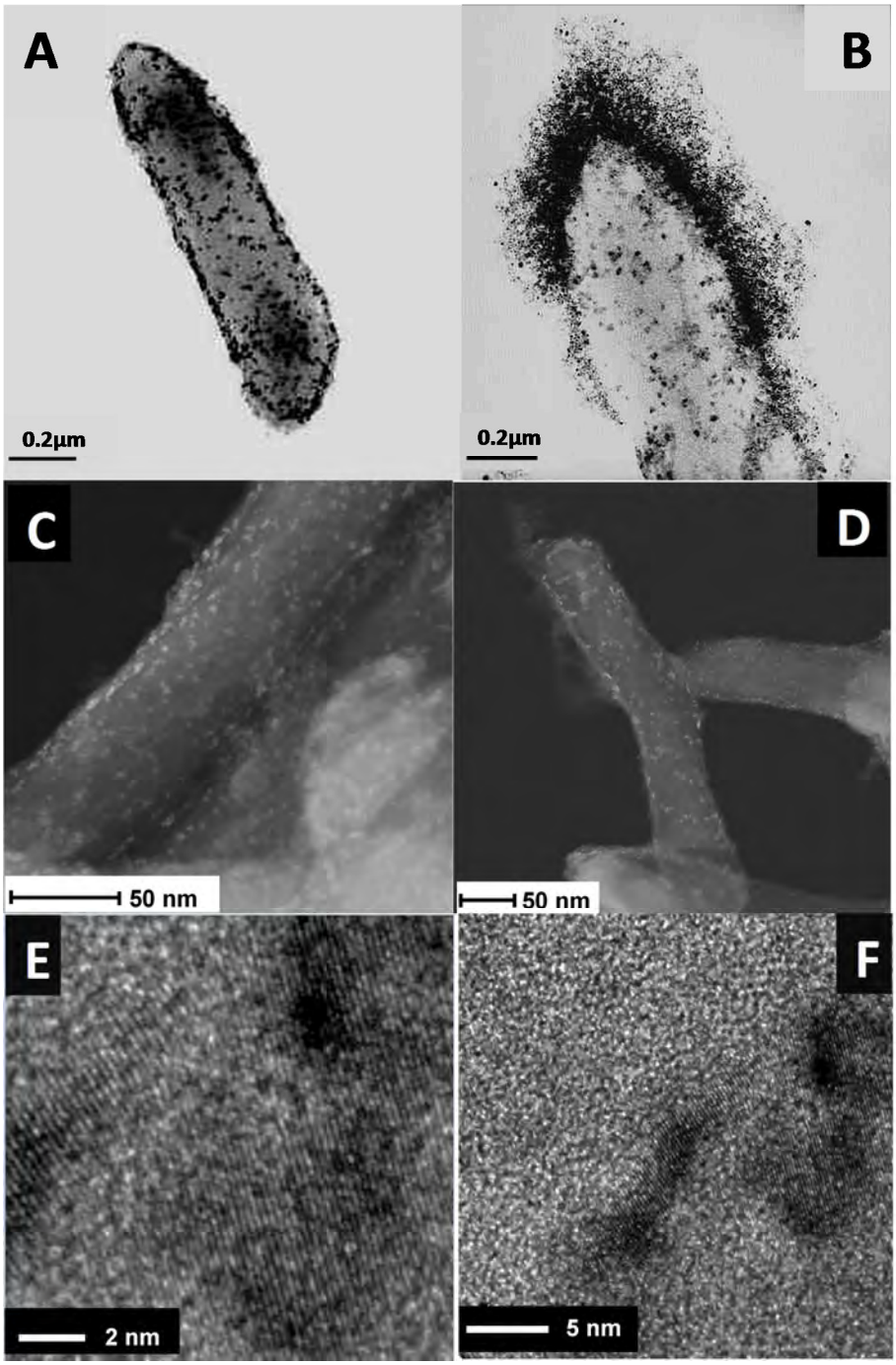

Figure 2.3. Pd(0) NPs covered from incubations of *G. sulfurreducens* provide with acetate as electron donor viewed by TEM in the absence (A) and in the presence of AQDS (B). HRTEM images and high angle annular dark field (HAADF) characterization of samples derived from incubation in the absence (C) and in the presence of AQDS (D). (E) and (F) HRTEM images showing planes of Pd(0) NPs.

Samples were also analyzed by field emission transmission electron microscopy using high angle annular dark field (HAADF) mode. By this method, it was possible to observe the nanoparticle distribution on the cell surface both in the presence and in the absence of AQDS (Figure 2.3C and 2.3D). Also elemental EDX microanalysis at areas of black precipitate confirmed that these were rich in palladium content (Figure 2.4A).

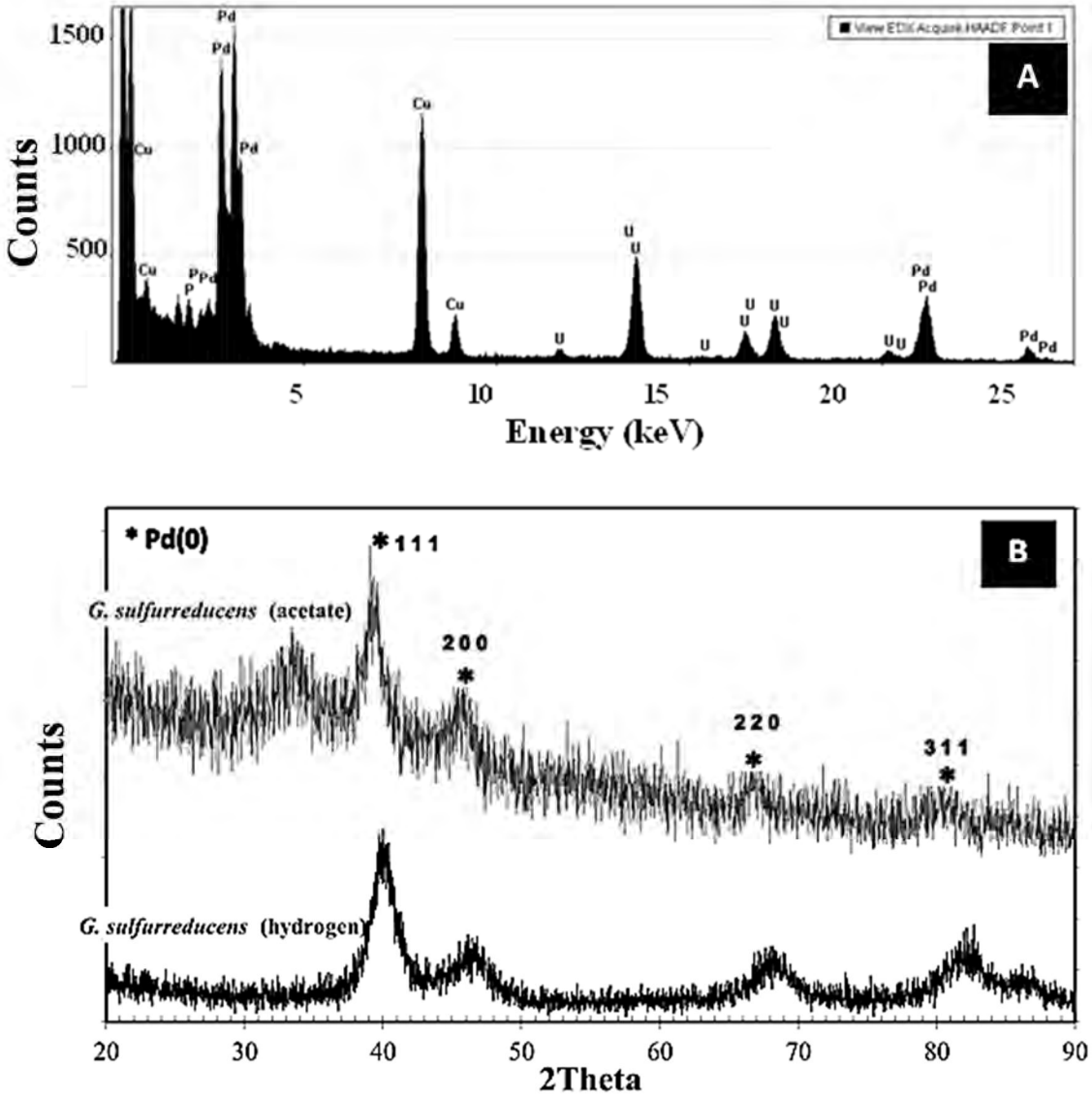

Figure 2.4 (A) EDX spectrum of precipitates deposited on *G. sulfurreducens*, uranium peaks are present due to the contrast method applied to stain cells (B) Comparison of XRD patterns corresponding to cells and black precipitates obtained from experiments carried out with acetate and hydrogen as electron donors.

By using HRTEM it was possible to confirm the crystalline nature of Pd NPs obtained. All Pd nanoparticles observed in HRTEM images showed lattice images indicating that they were single crystallites. The layers observed correspond to planes with spacing of d_{111}=0.228 nm (analyzed by DigitalMicrograph® 3.7.0) (Figure 2.3E and 2.3F). The analysis of Pd NPs confirmed their heterogeneous distribution on the cell surface (see Figure 2.3E and 2.3F).

XRD analysis confirmed the formation of Pd(0) NPs with an average diameter of 8.54 nm (analyzed by PowderCell® 2.4) in microbial incubations with both acetate and hydrogen as electron donors (Figure 2.4B). The XRD pattern showed five strong Bragg reflections at 2<theta> values around 40.11, 46.66, 68.13, 82.11 which correspond to planes (111), (200), (220) and (311) of a face-centered cubic lattice (fcc) (XRD pattern was indexed to ICDD card 89-4897 (fcc palladium syn)). HRTEM images, EDX and XRD pattern thus clearly showed that the Pd NPs were crystalline in nature.

2.5 Discussion

The present study demonstrates that *Geobacter sulfurreducens* is capable of using Pd(II) as terminal electron acceptor and is the first report on the reduction of this transition metal by this organism. The Pd(II) reduction by *G. sulfurreducens* resulted in the formation of nanoparticles of Pd(0), which became associated with the cell surface. The produced nanoparticles may have a potential use as nano-catalysts.

Regarding the metabolic capabilities of *G. sulfurreducens*, it has previously been reported that this strain can use a diversity of electron acceptors coupled to the oxidation of acetate or hydrogen under anaerobic conditions. Some examples of metals that *G. sulfurreducens* is able to reduce are Fe(III), Tc(VII), Co(III) and U(VI).[16–18,20,32,33] Furthermore, this organism can reduce humic acids and AQDS[34], which are reduced at the outer surface of this organism, where c-type cytochromes are intimately involved in the process.[19] Thus, the present study expands our understanding of the versatility of *G. sulfurreducens* in reducing several distinct terminal electron acceptors.

A recent report provides a comprehensive overview of bacterial species that are able to reduce Pd(II) to Pd(0)[13]. Most noteworthy are the Pd(II) reducing capacities of the sulfate-reducing bacterium, *Desulfovibrio desulfuricans*[15] and the iron-reducing bacterium *S. oneidensis.*[35] In both cases, NPs with remarkably narrow size distribution are formed within minutes at the outer surface of the bacterial cells.[14,36] Even though the two studies reported that high concentrations of Pd(II) were effectively reduced to Pd(0) (200 mg Pd(II) L^{-1} by *D. desulfuricans* and up to 1000 mg Pd(II) L^{-1} by *S. oneidensis*), there remains the challenge to decreased the size of the NPs in order to increase their catalytic activity.[37]

In the present study, the Pd(II)-reducing activity of *G. sulfurreducens*, in terms of mg Pd(II) reduced per liter per hour, was comparable to that of *S. oneidensis*. The ability of *G. sulfurreducens* to reduce metals and other terminal electron acceptors is due to its effective extracellular electron transfer processes accomplished through mechanisms, such as electrically conductive pili, cytochromes and multicopper proteins.[38–41] Additionally, the ability of *Geobacter* species to use quinone moieties as potential electron acceptors is noteworthy.[42] Therefore, palladium reduction rate (and parallel Pd(0) NPs production) by *G. sulfurreducens* can be enhanced by supplying catalytic quantities of redox mediators, such as AQDS, which promotes extracellular deposition of Pd(0) NPs with sizes ranging from 5 nm up to 15 nm in the present study. The effect of quinone redox mediators has previously been reported by Wang et al. (2011); who observed enhancement on the reduction of selenite (Se(IV)) and tellurite (Te(IV)) by *Escherichia coli* with the consequent formation and accumulation of extracellular precipitates of Se(0) nanospheres or Te(0) nanorods, respectively.

Addition of AQDS influenced the mechanism of Pd(0) NPs deposition, which occurred extracellular and on the cell surface of *G. sulfurreducens,* by allowing a long-distance electron transfer to Pd(II) promoted by the electron shuttling capacity of AQDS. The last observation implies that Pd(II) was reduced by AH_2QDS (reduced form of AQDS), which was microbially produced. Under these conditions, Pd(II) does not need to be in direct contact to the cell surface to be reduced to Pd(0), thus the prevalence of extracellular deposition of Pd NPs (Fig. 2.3B). Unlike other

microorganisms such as *Shewanella* species, which can secrete extracellular electron shuttles[43] *G. sulfurreducens* depends (when there has not been an electron shuttle added) on direct contact to Pd(II) (and other metals) to reduce it due to the location of cytochromes (extracellular space and outer membrane) necessary for electron transfer.[38]

Thus, the mechanism of Pd(II) reduction (when an extracellular electron shuttle is not present) can be described in two steps[15,44]: (1) adsorption or deposition of the metal on the cell, (2) enzymatic reduction and nucleation. This mechanism explains the results found for different CDW concentrations considering that cells represent the surface area available for Pd(0) NPs deposition. Namely, by providing a higher cellular density in microbial incubations, a faster reduction of Pd(II) and deposition of Pd(0) NPs is expected, a scenario which was shown to occur in the present study (Table 2.2). Although the exact mechanisms is not yet understood, it can be inferred from the standard reduction potentials values that the order in which the Pd species are reduced is $PdOH^{+}$> Pd^{2+}> $PdCl^{+}$>$PdCl_{2(aq)}$>$PdCl_4^{2-}$>$PdCl_3$> $Pd(NH_3)^{2+}$> $Pd(NH_3)_2^{2+}$> $Pd(OH)_{2(s)}$> $Pd(NH_3)_3^{2+}$> $Pd(NH_3)_4^{2+}$ (see Table 1). However, the reduction mechanism and contribution of each of these species on this process require further studies.

The promotion of extracellular nucleation of Pd(0) NPs by the role of AQDS as redox mediator represents an attractive alternative to facilitate the recovery of produced nano-catalyst from microbial incubations because a significant fraction could easily be separated from microbial cells.

Another advantage of the application of *G. sulfurreducens* with redox mediators to produce Pd(0) nano-catalyst is the use of acetate as electron donor, which is economic and safer than hydrogen. Hydrogen has been used as electron donor with hydrogenotrophic mircoorganisms[37]. In fact, *G. sulfurreducens* is the first acetoclastic Pd(II)- reducing microorganism reported in the literature to the best of our knowledge.

Bio-palladium produced by different microbial species has been proposed as a nano-catalyst to promote the biodegradation of priority contaminants, such as: chlorinated, iodinated and brominated organic compounds (e.g., chlorinated ethane, diatrizoate),

through hydrodehalogenation; oxyanions (e.g., perchlorate, chlorate, nitrate) by hydrodeoxygenation and azo-dyes through N-N hydrogenolysis.[9] The application of bio-palladium includes micropollutant removal, such as pharmaceuticals and pesticides, which constitute a major concern in wastewater treatment.[2,37,45]

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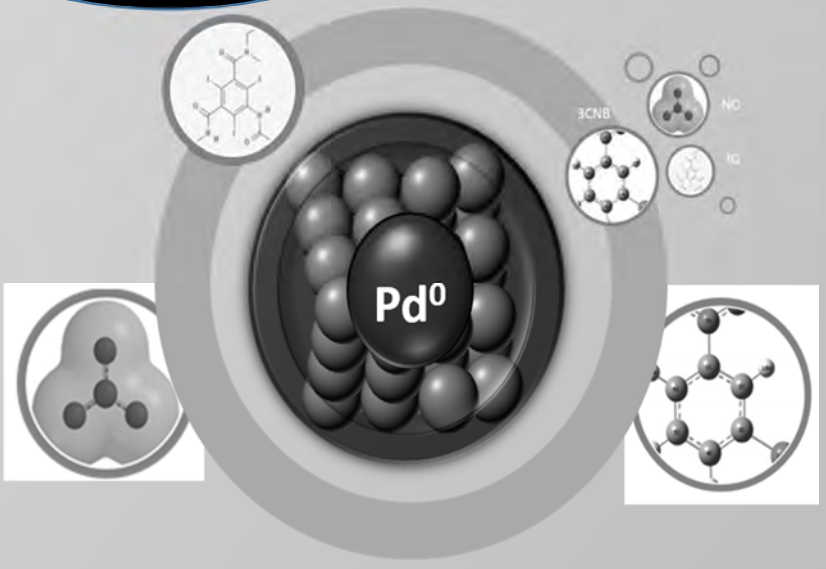
CHAPTER 3

Direct and quinone-mediated palladium reduction by *Geobacter sulfurreducens*: mechanisms and modeling



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3.1 Abstract

Palladium(II) reduction to Pd(0) nanoparticles by *Geobacter sulfurreducens* was explored under conditions of neutral pH, 30 °C and concentrations of 25, 50 and 100 mg Pd(II) L^{-1} aiming to investigate the effect of solid species of palladium on their microbial reduction. The influence of anthraquinone-2,6-disulfonate is reported to enhance the palladium reaction rate in an average of 1.7-fold and its addition is determining to achieve the reduction of solid species of palladium. Based on the obtained results two mechanisms are proposed: 1) direct, which is fully described considering interactions of amide, sulfur and phosphoryl groups associated to proteins from bacteria on palladium reduction reaction, and 2) quinone-mediated, which implies multi-heme c-type cytochromes participation. Speciation analysis and kinetic results were considered and integrated into a model to fit the experimental data that explain both mechanisms. This work provides elements for a better understanding of direct and quinone-mediated palladium reduction by *G. sulfurreducens*, which could facilitate metal recovery with concomitant formation of valuable palladium nanoparticles in industrial processes.

3.2 Introduction

There is an increasing demand of precious metals due to their use as catalysts for several industrial applications. Hence, their recovery became of foremost importance and many efforts have been focused on developing new strategies for metals recycling.[1] Particularly, palladium recovery is necessary as it is the most widely used catalyst and its price has greatly oscillated in the last decade because of a variable supply and demand. In addition, palladium is being required in the form of nanoparticles (NP's), a reason for what new methods, such as biological synthesis using bacteria, have been explored. The use of bacteria presents advantages for both metal recovery and NP's synthesis as it is considered to be green, tunable, affordable and scalable.

Several bacteria species have been proved to be able to reduce Pd(II) to Pd(0), but reports elucidating the mechanisms involved are scarce. In general, the microbial reduction of Pd(II) has been proposed to involve sorption of palladium to the cell,

Pd(0) nucleation and autocatalytic reduction of Pd(II) by the already formed Pd(0).[2] However conditions prevailing during the microbial palladium reduction process influence the reported findings and their interpretation. Most studies have been evaluated at low pH values[2-4] and, under these conditions, evidence has been provided indicating that reduction on microbial surfaces is possibly mediated by a non-enzymatic mechanism.[2] Solution pH usually plays a major role since this affects the solution chemistry of metals and, most importantly, the activity of functional groups of macromolecules such as phospholipids, lipoproteins, lipopolysaccharides, and proteins present in the membrane of bacteria. Those macromolecules from biomass provide available ligand groups on which metal species are expected to bind, some examples are: carboxylate, thiolate, phosphate and nitrogen containing groups.[5] Preferences of metal species for a specific ligand-binding site depend on their chemical coordination characteristics. Techniques such as Fourier transform infrared (FTIR) spectroscopy offer highly specific information about chemical composition of cell samples and, depending on the technique employed, only minimal sample preparation is necessary.[6]

For metals, the pH prevailing in the reaction system strongly influences the speciation and availability of metal ions.[7,8] Metal speciation also affects the preference for ligand-binding site and this could change for different ionic species.[5]

Another factor that directly impacts the mechanism of metal reduction is the presence of a redox mediator. It has been reported that different quinone redox mediators enhanced the reduction rate and promoted extracellular formation of metals NP's.[8, 9]

The aim of the present study was to elucidate the mechanisms involved during the microbial reduction of Pd(II) to produce Pd(0) NP's by *Geobacter sulfurreducens* . The effect of the redox mediator, anthraquinone-2,6-disulfonate (AQDS), on the microbial reduction of Pd(II) was also evaluated and a mathematical model has been developed to describe the microbial process.

3.3 Material and methods

3.3.1 Microorganism culture

Strain *Geobacter sulfurreducens* PCA (DSM 12127; ATCC51573) was routinely cultured anaerobically in acetate:fumarate medium as previously described at 30 °C.[10,11]

3.3.2 Microbial reduction of Pd(II) by *G. sulfurreducens*

For experiments of microbial reduction of Pd(II), late logarithmic phase cultures of *Geobacter sulfurreducens* were used. The protocol consisted of harvesting cells by centrifugation and washing with a sterilized, osmotically balanced buffer.[8,12] The reaction buffer composition[12] was (g L^{-1}): $NaHCO_3$ (2.5), NH_4Cl (0.25), $NaH_2PO_4 \cdot H_2O$ (0.006), KCl (0.1)) The reduction experiments were performed in glass serum bottles capped with inert viton stoppers with 100 mL of final total volume of anaerobic basal medium flushed with N_2/CO_2 (80%/20%). Cell suspension and acetate were added to yield a concentration of 800 mg L^{-1} cell dry weight (CDW) and 5 mM, respectively. Depending on the experimental set-up, an appropriate volume of an anoxic Na_2PdCl_4 (Sigma-Aldrich) stock solution was added to give one of three different final Pd(II) concentrations, 25, 50 and 100 mg Pd(II) L^{-1}. For the experiments carried out to evaluate the effect of the redox mediator, AQDS, the basal medium was supplemented with 100 µM of AQDS. All serum bottles were then incubated for 24 h at 30 °C. Control experiments were also incubated under the same conditions, but in cell-free media. All experimental treatments were set up in triplicate and sampled at specific time intervals. Reduction kinetics was followed by analyzing Pd concentration and acetate consumption. The speciation analyses of Pd were performed using Visual MINTEQ® software ver. 3.0.

3.3.3 Analytical techniques

To document microbial reduction of Pd(II) and acetate consumption, samples were separately analyzed by inductively coupled plasma-optic emission spectroscopy (ICP-OES, Varian 730-ES) and capillary electrophoresis (Agilent 1600A, Waldbronn, Germany), respectively. For ICP-OES analysis, samples of 5 mL were filtered (0.22

μm Millipore, Bedford, USA) and both fractions obtained (filtrate and filter) were analyzed. The filter containing the retained fraction was treated with aqua regia (boiled during 2 h), and the solubilized fraction of palladium obtained was diluted before analysis. These analyses were carried out in order to evaluate, during the incubation time, the palladium content associated to biomass (filter) and palladium remaining in solution (filtrate).

Acetate concentration was determined by capillary electrophoresis using a fused silica capillary column (Agilent, id 50 μm, 80.5 cm long, effective length 72 cm) and basic anion buffer (Agilent, pH 12.1). Quantification was done by comparison with high purity standard. Conditions of analysis were temperature 20°C, voltage -30 kV, pressure injection of 300 mbar for 6 s, signal wavelength set a 350 nm with a reference at 230 nm. Detection was carried out with indirect UV using a diode-array detector. Prior each run, it was performed a 4 min buffer flush at 1 bar.

The zeta potential of cells, before and after palladium reduction, and of microbially-produced NP's was analyzed in a Microtrac® Zetatrac model PMX 300. All the samples were prepared in the reaction buffer described above and analysis were conducted at room temperature and pH 7. Palladium NP's produced by *G. sulfurreducens* were obtained by an acetone treatment in order to remove the cells as described by [1] et al.[13]

3.3.4 FT-IR spectra

Analysis of cell samples before and after Pd(II) reduction assays were carried out in order to identify variations in functional groups. Cells were lyophilized by freezing at – 80 ° C and drying under vacuum for 48 h.[2] Lyophilized samples were mixed with KBr at a ratio of 1:99% (w/w) and then pressed into a pellet form for FT-IR analysis. The spectra (64 scans, range 400-4000 cm^{-1}) were recorded on a Thermo-Scientific FTIR (Nicolet 6700 model) spectrophotometer.

3.3.5 Transmission electron microscopy

Samples from different assays were prepared for transmission electron microscopy (TEM) by fixation in 3% glutaraldehyde and staining with 2% uranyl acetate as

previously described.[8] Observations were carried out with a JEOL electron microscope model 200 CX, operated under standard conditions at 80 kV.

3.3.6 X-ray diffraction analysis

Analysis of the palladium NP's obtained by *G. sulfurreducens* cells was carried out in an X-Ray diffractometer Bruker D8 Advance. Samples were prepared as described previously by Lloyd.[4] X-ray diffraction (XRD) patterns were recorded from 20°-90° 2θ with a step time of 2 s and step size of 0.01° 2θ.

3.3.7 Mathematical model and equations solving

Based on the results obtained aspects that play an important role in the microbially driven reduction kinetics were considered in a mathematical model to explain the different effects in the process. These considerations are analogous to those reported by [2]who explored biologically mediated reduction of thin layers of poorly crystalline iron oxides by *G. sulfurreducens* with and without an electron shuttle. Such that, the proposed model considers: (a) addition of AQDS as redox mediator that enhances the reduction rate constant, where the rate of AQDS reduction is proportional to biomass and to the concentration of AQDS added; (b) a fraction that defines the amount of soluble palladium available to be reduced by microorganisms which is calculated according to speciation analysis; (c) an effect of the surface area exposed by bacteria and the external mass-transfer coefficient on the reduction rate of palladium; (d) *G. sulfurreducens* can reduce palladium by two ways when AQDS is present in the medium, directly (palladium contact with cell membrane cytochromes) and indirectly (via electron shuttling) through AQDS; (e) for one mol of AH_2QDS (reduced form of AQDS) oxidized, one Pd(II) mol is reduced; (f) biomass growth is not considered but inhibition or decay is taken into account.

Equation (3.1) represents the proposed model for the reduction of palladium via direct contact (without AQDS), where K is a reduction constant; f represents the fraction of soluble palladium available; X_s is a saturation function calculated as in equation (3.2), where X is the biomass and K_{xs} is a constant with the same units (cells per mg of palladium); S represents the product of the liquid-solid external mass

transfer coefficient and the surface area of biomass; C is the concentration of palladium and R is the parameter that represents a delay factor (which have an impact on palladium reduction) which considers cell decay or inhibition (this factor implies palladium demand for cell decay). The model for the reduction of palladium in the presence of AQDS includes terms proposed by MacDonald et al., [14] and equations (3.3), (3.4) and (3.5) represent the complete model. Where μ is the reduction constant for AQDS, α is the rate constant for AH_2QDS-palladium; [AQDS] and [AH_2QDS] are the corresponding values of concentration which were estimated by fitting the experimental data, the values of parameters μ and α were initially proposed according to the values found by MacDonald et al.[14]

The model equations proposed to fit the experimental data obtained from microbial reduction experiments were solved by using software Matlab7. Equations were coded as ordinary differential equations and integrated using the solver ode45 and ode15, which uses a variable step Runge-Kutta method to numerically solve differential equations.

Parameter estimation was carried out using Matlab optimization function lsqcurvefit in order to minimize the difference between real data and modeled results. The lsqcurvefit optimization tool from Matlab allows solving nonlinear curve-fitting problems in the least-squares sense by finding coefficients that best-fit the equation provided. Parameter searching was accomplished using the large-scale, trust-region reflective Newton method.

$$\frac{dPd}{dt} = K\, f\, X_s\, S\left(C_0 - C\right) - R\, X_s \tag{3.1}$$

$$X_s = \frac{X}{K_{xs} + X} \tag{3.2}$$

$$\frac{dPd}{dt} = K\, f\, X_s\, S\left(C_0 - C\right) + \alpha\ \left[AH_2QDS\right]\left(C_0 - C\right) - R\, X_s \tag{3.3}$$

$$\frac{d\left[AH_2QDS\right]}{dt} = \mu\, \mathrm{X}\left(C_0 - C\right)[\mathrm{AQDS}] - \alpha\ \left[AH_2QDS\right]\left(C_0 - C\right) \tag{3.4}$$

$$\frac{d\left[AQDS\right]}{dt} = -\mu\, \mathrm{X}\left(C_0 - C\right)[\mathrm{AQDS}] + \alpha\ \left[AH_2QDS\right]\left(C_0 - C\right) \tag{3.5}$$

The parameter estimation was done by first calculating the parameter corresponding to the data series without AQDS, then the fitting parameters were used as inputs into the model used for the experimental data of the reduction of palladium with AQDS as redox mediator.

3.4 Results

3.4.1 Speciation of palladium during its microbial reduction

In order to determine the main palladium species dominating the reaction system during their microbial reduction by *G. sulfurreducens* speciation analysis were conducted. Results showed, in all cases, that $Pd(NH_3)_4^{2+}$ was the predominant soluble species representing 99.08, 66.16 and 33.08% of the total palladium concentration at initial concentrations of 25, 50 and 100 mg Pd(II) L^{-1}, respectively (Table 3.1). The second soluble species in abundance was $Pd(NH_3)_3^{2+}$ representing 0.89, 0.65 and 0.33% of the total palladium concentration under the same conditions. The species $Pd(OH)_{2(s)}$ was found to be the main solid form of palladium in the medium and its presence was related to the initial concentration of Pd(II) used.

Table 3.1 Distribution of palladium species and kinetic results for the reduction of Pd(II) by *G. sulfurreducens* after 24 h of incubation for different Pd(II) concentrations

Pd initial concentration (mg L^{-1})	Predominant species (mg L^{-1})		Solid formation (mg L^{-1})	Pd remaining in solution (mg L^{-1})	
	$Pd(NH_3)_4^{+2}$	$Pd(NH_3)_3^{+2}$	$Pd(OH)_{2(s)}$	Acetate	+AQDS
25	24.77	0.223	0.08	3.52±0.77	0.39±0.23
50	33.08	0.33	16.58	15.13±1.94	0.15±0.04
100	33.08	0.33	66.57	61.30±0.81	0.01±0.01

According to the results summarized in Table 3.1, it was determined that for a concentration of 25 mg Pd(II) L^{-1}, no significant formation of the precipitate $Pd(OH)_{2(s)}$ was produced (0.02% of the total palladium concentration) while for the

concentrations of 50 and 100 mg Pd(II) L^{-1} the solid product represented 33.2 and 66.6% of the total concentration of Pd(II) in the reaction medium.

3.4.2 Microbial reduction of Pd(II) by *G. sulfurreducens*

Results from microbial incubations without AQDS demonstrated that *G. sulfurreducens* can reduce Pd(II) to Pd(0) with acetate as electron donor at concentrations of 25, 50 and 100 mg Pd(II) L^{-1}. Nevertheless, palladium reduction was incomplete when solid species of palladium were present in significant amounts (experiments with 50 and 100 mg Pd(II) L^{-1}) since Pd reduction stopped when the soluble palladium species were depleted (Table 3.2).

Table 3.2 Kinetic values obtained for the different experimental conditions evaluated.

	25 mg Pd L^{-1}		50 mg Pd L^{-1}		100 mg Pd L^{-1}	
	Acetate	+AQDS	Acetate	+AQDS	Acetate	+AQDS
% Pd reduced	83.01±2.3	97.62±0.9	62.12±1.4	98.52±1.3	38.70±3.2	99.98±0.02
Specific reduction rate (mg Pd g^{-1} CDW h^{-1})	17.06	23.05	16.35	34.75	20.63	33.71

For AQDS-amended cultures it was found that the presence of solid species of palladium, such as $Pd(OH)_{2(s)}$, was not a hindrance for Pd(II) reduction. In addition, reduction kinetic parameters showed that palladium specific reduction rate increased 1.35−, 2.13− and 2−fold when AQDS was present for experiments performed with 25, 50 and 100 mg Pd(II) L^{-1}, respectively, as compared with microbial incubations lacking added AQDS .

Palladium-covered cells observed by means of TEM showed that extracellular formation of NP's was promoted when AQDS was present and XRD analysis

confirmed the nature of NP's obtained as Pd(0) for the experiments with and without AQDS (Figure 3.1).

According to the results obtained from Pd(II) reduction kinetics (Table 3.1), AQDS played two important roles: enhanced the microbial reduction of Pd(II) by *G. sulfurreducens* and facilitated the reduction of solid species of palladium ($Pd(OH)_{2(s)}$).

For sterile controls lacking cells, no changes were found on acetate and palladium concentrations (with or without AQDS). The pH was measured after the experiments and no change was detected (7.1±0.1) in all experimental treatments.

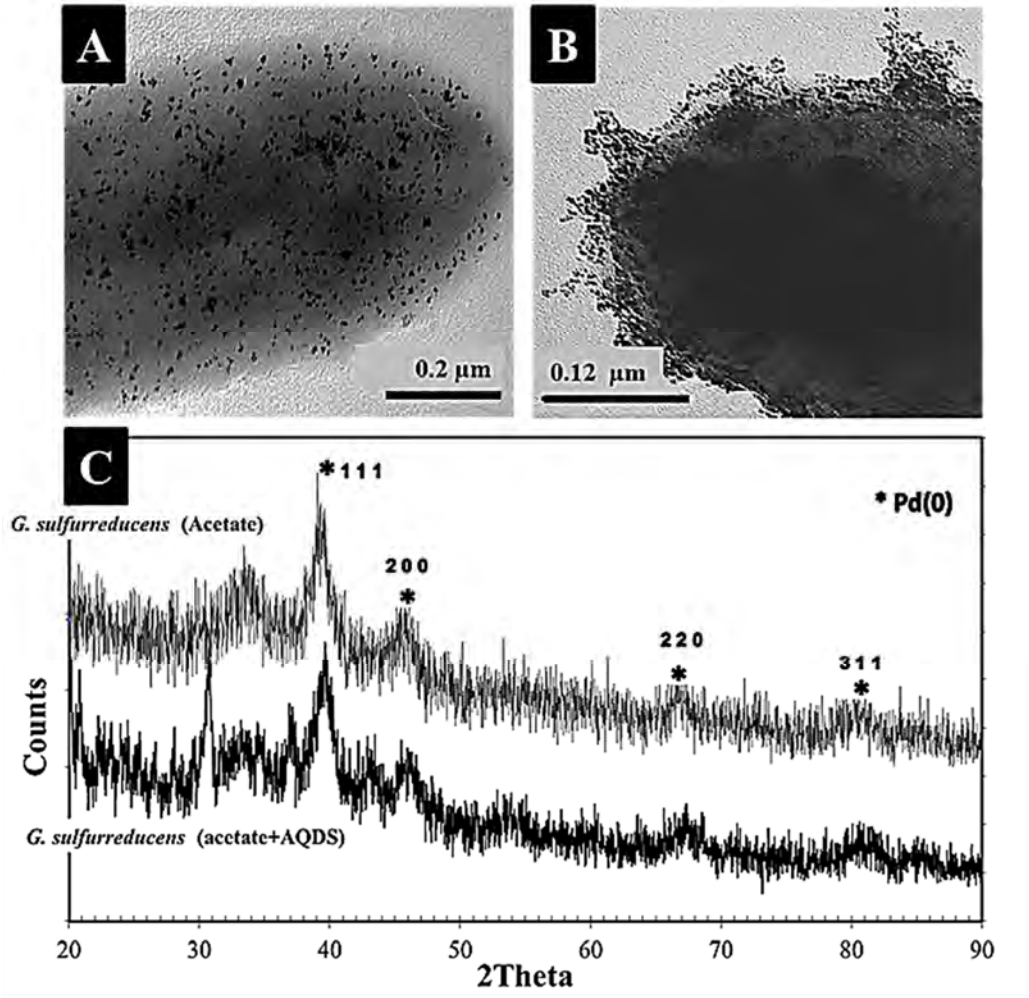

Figure 3.1 Pd(0) NPs covered from incubations of *G. sulfurreducens* provided with acetate as electron donor viewed by TEM in the absence (A) and in the presence of AQDS (B) (for a concentration of 50 mg Pd(II) L^{-1}). (C) Comparison of XRD patterns corresponding to cells and black precipitates obtained from experiments carried out with acetate as electron donor in presence and absence of AQDS.

3.4.3 Zeta potential analysis

Zeta potential values of analyzed samples are summarized in Figure 3.2. Negative zeta potential values are reported both for *G. sulfurreducens* cells before the reduction experiment and for palladium NP's, -3.8± 0.26 mV and -39.82 ± 6.32 mV, respectively.

Cells of *G. sulfurreducens* after palladium reduction, without AQDS had similar zeta potential as *G. sulfurreducens* cells (Figure S1). On the contrary, the zeta potential value of sample corresponding to cells of *G. sulfurreducens* after palladium reduction with AQDS, became more negative (-30.83 ± 7.36 mV) than *G. sulfurreducens* cells not exposed to Pd(II). Hence, the zeta potential in this case may represent the value of extracellular palladium NP's rather than to the surface of cell membrane of *G. sulfurreducens*. This result is consistent with the effect of AQDS on the promotion of extracellular formation of palladium NP's (Figure 1). Moreover, the increase in the negative zeta potential value could be associated to a higher production of Pd(0) NP's.[15] To support this reasoning, the palladium mass deposited per unit area was calculated based on both the results obtained from microbial Pd(II) reduction experiments and considering a cell as a cylinder with diameter of 0.5μm and length of 2.5 μm.[16] Results evidenced that the mass of palladium per unit area (mg Pd μm^{-2}) increased 1.2, 1.5 and 2.3-fold when using AQDS for experiments performed at concentrations of 25, 50 and 100 mgPd(II) L^{-1}, respectively, as compared to experiments lacking AQDS and these results are in agreement with the interpretation of the zeta potential value obtained.

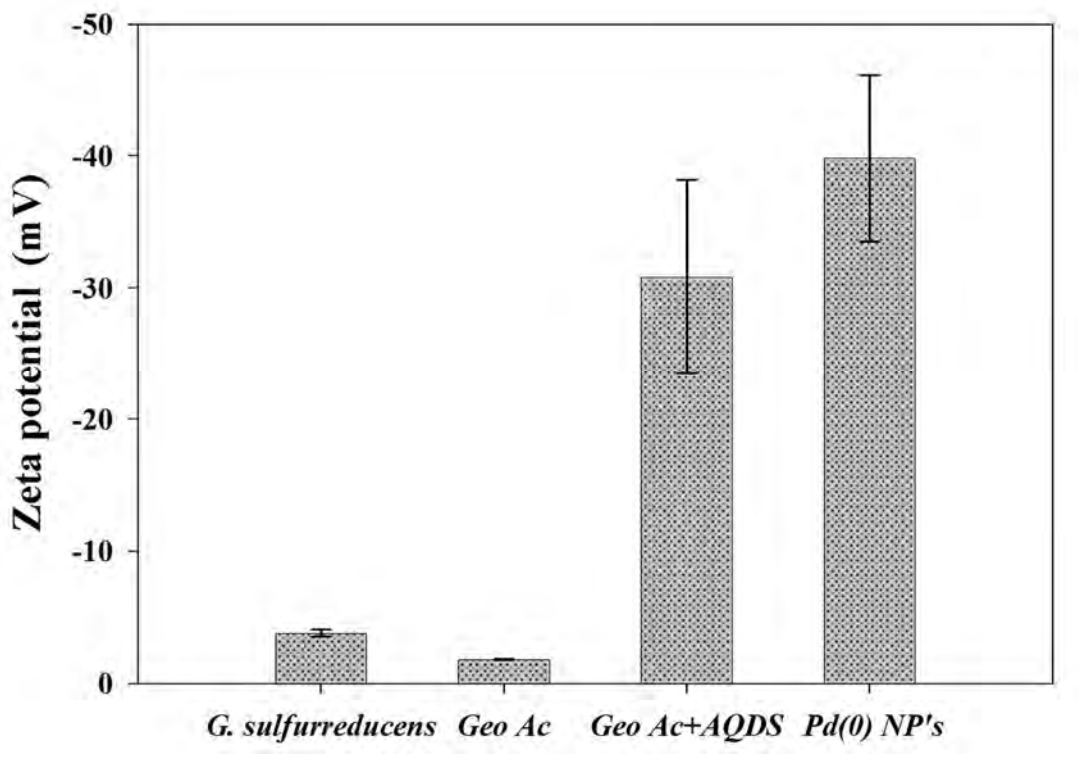

Figure 3.2 Zeta potential values of *G. sulfurreducens* cells before (G. sulfurreducens), after palladium reduction (25 mg Pd L^{-1}) in absence (Geo Ac) and presence of AQDS (Geo Ac+AQDS) and sole palladium nanoparticles (Pd(0) NP's). Zeta potential of samples was measured in medium reaction, pH 7.1±0.1.

3.4.4 FT-IR spectra

The composition of Gram-negative bacteria, such a *G. sulfurreducens*, is complex, compared to Gram-positive bacteria, because in addition to the cell wall composed of peptidoglycan, cells present an outer membrane that contains phospholipids, lipoproteins, lipopolysaccharides, as well as structural and enzymatic proteins.[17] The nature of these cellular components is translated in the presence of several functional groups: carboxyl, phosphonate, amine, etc., and considering that the first component that comes into contact with metal ion is the bacterial cell wall, FT-IR analysis were carried out to identify and compare functional groups presents in cells of *G. sulfurreducens* before and after Pd(II) reduction and, based on this information, postulate its contribution during microbial palladium reduction. In order to analyze in

detail the information provided by FT-IR spectra, it was divided in two sections of interest where differences were detected, as it is shown in Figure 3.3.

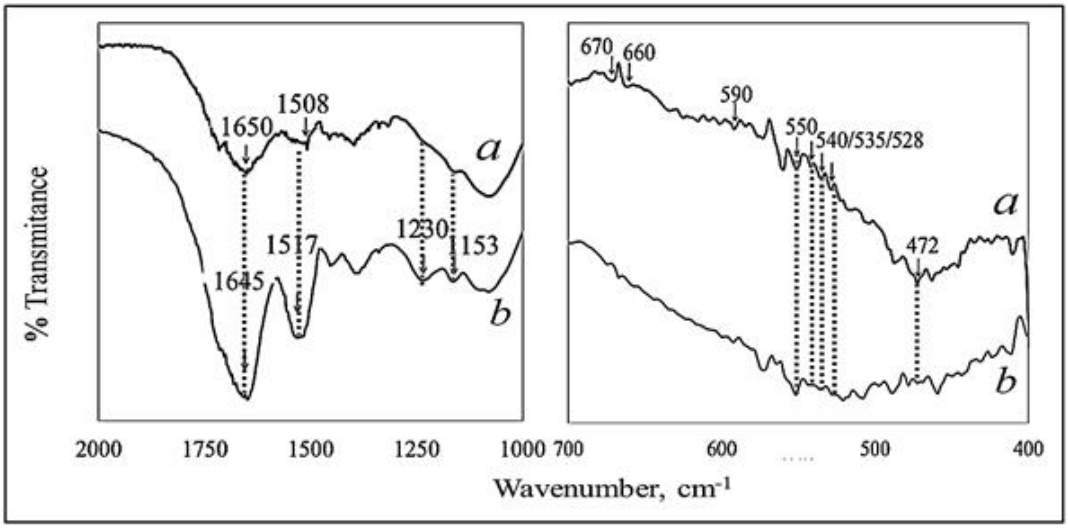

Figure 3.3 FT-IR spectra of *Geobacter sulfurreducens* cells before (a) and after palladium reduction experiment (b).

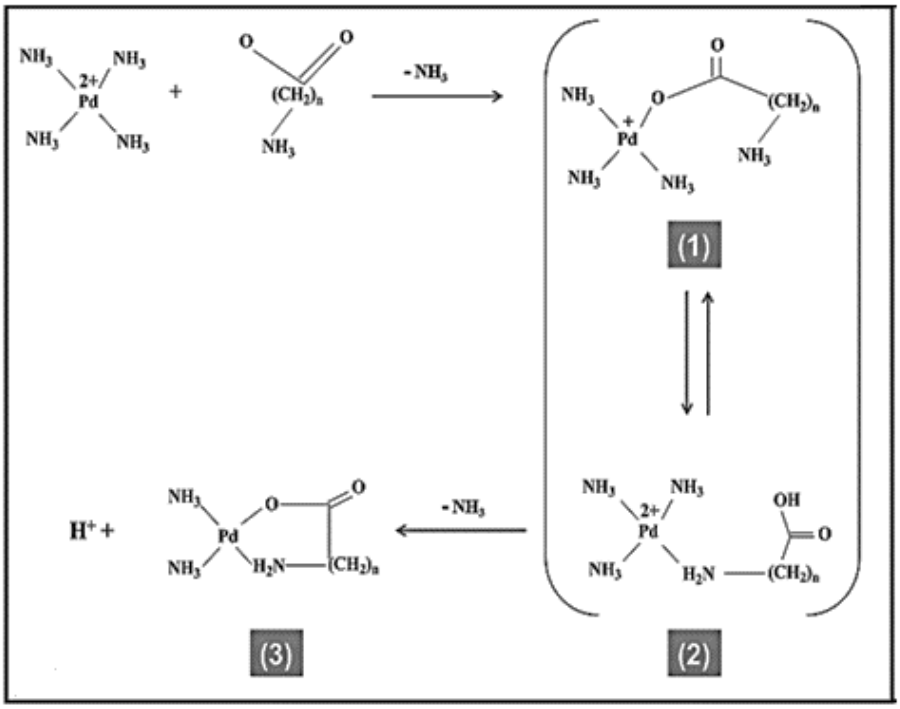

Figure 3.4 Ligand substitution reactions involved in the $[Pd(NH_3)_4]^{2+}$ interaction with N-terminal group of a model compound structure.

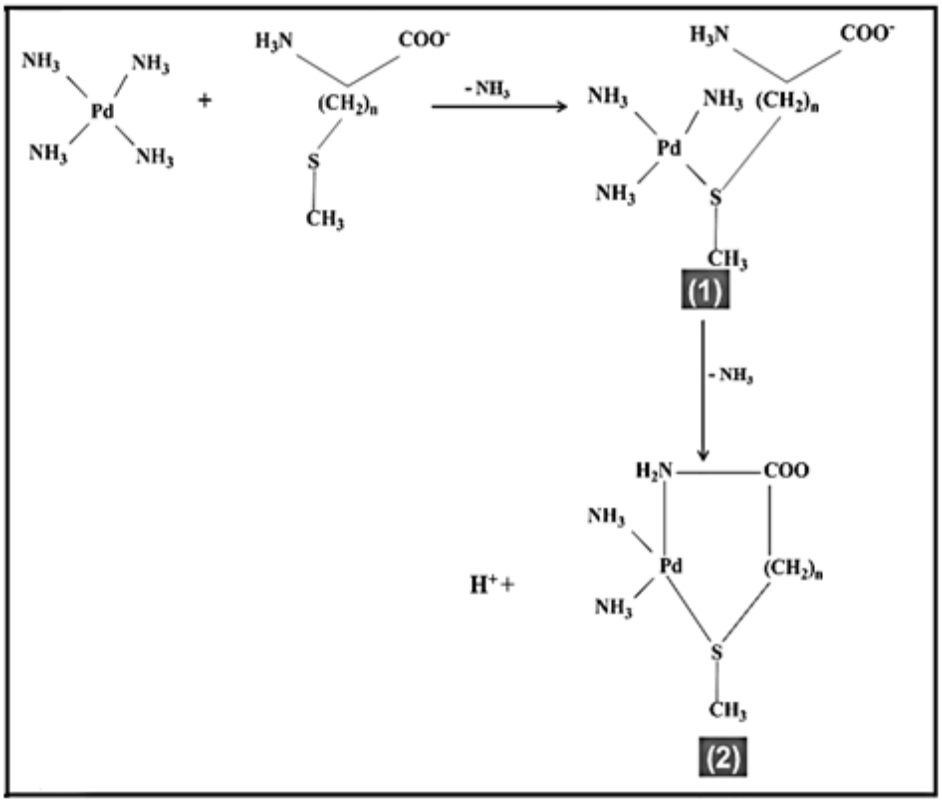

Figure 3.5 Ligand substitution reactions involved in the $[Pd(NH_3)_4]^{2+}$ interaction with with S-containing group of a model compound structure.

Peaks in the mid-IR region of 1700-1500 cm^{-1} correspond to the presence of amide group CO·NH. Noticeable changes were observed in bands of amide I and amide II in the spectra. The peaks observed in both spectra (*G. sulfurreducens* cells before and after the Pd(II) reduction assay, lines (a) and (b) in Figure 3.3) at 1645 and 1650 cm^{-1} correspond to the main strong amide I band (~80% CO stretching, ~10% CN stretching, ~10% NH bending vibration) (Silverstein et al., 2005) while absorption at 1508 and 1517 cm^{-1} is associated to amide II band (~60% NH bending vibration, ~40% CN stretching).[18] Peaks at 1230 and 1150 cm^{-1} (spectra b in Figure 3.3) were also observed and assigned to PO_2^- symmetric vibration and C-O stretching of CR_2OH of lipids.[19]

Bands present in the far-IR region, such as the peaks at 670-660 cm^{-1} attributed to out of plane N–H wagging for hydrogen bonded amides, are apparently increased in

intensity after Pd(II) reduction. Bands corresponding to cyano complexes due to Metal-Carbon (M-C) stretching are observed in the region 600-350 cm^{-1} and due to Metal-CN (M-CN) deformation in the region 500-350 cm^{-1}. Metal-ammine complexes also have a number of weak to medium intensity bands in the region 535-275 cm^{-1} and Pd-C stretching of square planar complexes appear around 535 cm^{-1}. Changes observed between spectra may be attributable to interactions of groups present in the cell wall and palladium. Additionally, two peaks at 2370 and 2306 cm^{-1} (data not shown) corresponding to simple nitrile complexes[19] were also detected.

Comparing the spectra of cells before and after the palladium reduction it can be observed a significant intensity variation of peaks attributed to amide I, amide II, N-H wagging for hydrogen bonded amides and PO_2^- symmetric vibration and C-O stretching of CR_2OH of lipids, which might suggest the participation of amine and phosphoryl groups during microbial reduction of palladium.

3.4.5 Modeling of microbial reduction of palladium

Table 3.2 shows the model parameter estimates and Figure 3.6 shows the graphs for model fitting results and experimental data. Data series were fitted with a correlation factor greater than 0.97 (see Table 3.3).

The model parameter results obtained for the rate constant (K) for palladium reduction confirmed that the reduction reaction increased, in average, 1.4-fold when AQDS is present (compared with experimental data indicating an average increase of 1.7-fold). The value of the parameter R (delay factor for palladium reduction) increases as it does the concentration of the solid palladium species $Pd(OH)_2$. Values for R parameter for treatments without AQDS added were, in average, 2.7-fold higher than the corresponding R values estimated for treatments with AQDS, which reflect the difficulty for *G. sulfurreducens* to access solid species of palladium and reduce them. Factor S showed a good response when data corresponding to a different surface cell area were tested (data not shown) and the numerical value calculated for the liquid-solid external mass transfer coefficient might be considered only for these experimental conditions (calculated as 5.17×10^{-5} m h^{-1}, considering the cell as a cylinder with diameter of 0.5μm and length of 2.5 μm.[16]

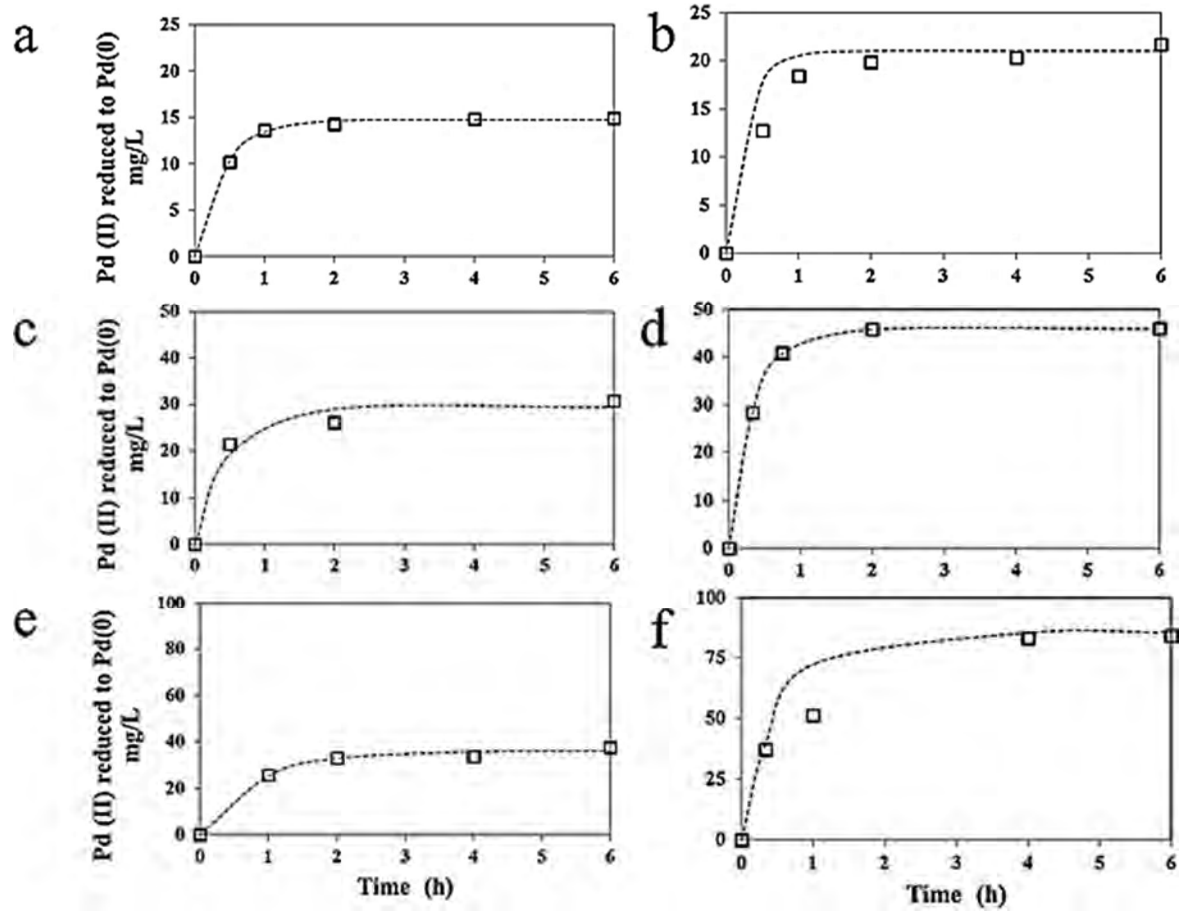

Figure 3.6 Kinetic evolution of Pd reduction of experimental data (□) and modeled results (---) for the experiments lacking AQDS for a concentration of 25 mg Pd(II) L^{-1} (a), 50 mg Pd(II) L^{-1} (c) and 100 mg Pd(II) L^{-1} (e), and experimental data for incubation with AQDS added and concentrations of 25 mg Pd(II) L^{-1} (b), 50 mg Pd(II) L^{-1} (d) and 100 mg Pd(II) L^{-1} (f).

Table 3.3 Model parameter estimates for different palladium concentrations in presence and absence of AQDS

Parameter	Values					
	25 mg Pd L^{-1}		50 mg Pd L^{-1}		100 mg Pd L^{-1}	
	Acetate	+AQDS	Acetate	+AQDS	Acetate	+AQDS
f [a]	0.99	1	0.668	1	0.334	1
X^a (cell mg^{-1} Pd)	3.2×10^{10}	3.2×10^{10}	1.6×10^{10}	1.6×10^{10}	8×10^{9}	8×10^{9}
K (mg Pd $L^{-1})^{-1}$	13.73	18.23	14.725	20.86	19.06	25.69
K_{Xs} (cell mg^{-1} Pd)	1.8×10^{8}	1.8×10^{8}	1.4×10^{8}	1.4×10^{8}	1.1×10^{7}	1.1×10^{7}
R (mg Pd $cell^{-1}$ h^{-1})	21.186	9.19	33.986	12.19	69.838	28.19
μ (L $cell^{-1}$ h^{-1})		6.26×10^{-11}		6.26×10^{-11}		6.26×10^{-11}
S (d^{-1})	0.178	0.178	0.178	0.178	0.178	0.178
α (L^2 h^{-1} mg^{-1} AH_2QDS mg^{-1} Pd)		1.199×10^{-2}		1.199×10^{-2}		1.199×10^{-2}
R^2	0.9992	0.9712	0.9868	0.9973	0.9966	0.9715

3.5 Discussion

The results obtained in this study demonstrate that *Geobacter sulfurreducens* can reduce both soluble and insoluble species of (Pd(II) by means of two mechanisms: (i) direct contact typically involving reduction by outer membrane c-type cytochromes; and (ii) indirectly by using AQDS as an electron shuttle. These findings are consistent with similar reports regarding Fe(III) species.[20-23]

Several reports on *Geobacter sulfurreducens* and other dissimilatory metal-reducing bacteria have demonstrated that multiheme c-type cytochromes (c-Cyts) are responsible for the ability of extracellular electron transfer.[24,25] Furthermore, the genome of *G. sulfurreducens* predicted 111 c-Cyts, and it has been reported that some outer membrane c-Cyts, such as OmcS and OmcB, required for electron transfer to insoluble Fe(III), are more highly expressed during growth on Fe(III) oxide than with fumarate.[26,27] This could explain the results obtained for the palladium reduction experiments in the absence of AQDS in which the reaction stopped when soluble species of palladium had been depleted.

Certain redox-active metals can be respired outside the cell even though the metal species are solubilized; this pathway is influenced by the nature of the respired metal species since reduced forms of some metals precipitate due to their low solubility (for example Se and the radionuclides U and Tc).[21,24,28,29] Palladium followed this behavior and *G. sulfurreducens* reduces soluble species of Pd using acetate as electron donor (see reaction c) and it occurs in the presence or in the absence of AQDS. Nevertheless, addition of AQDS enhances the rate of metals reduction as it has been previously proved.[8,9] Furthermore, results found in the present study evidenced that the presence of AQDS is essential for the reduction of insoluble species of palladium. Reaction (3.6) represents the step of microbial AQDS reduction and reaction (3.7) represents the abiotic reduction of palladium by AH_2QDS. Thermodynamic considerations indicate that Gibbs free energy (ΔG) for the global reaction (3.8) is the same in the presence and in the absence of the redox mediator, but in the latter case the cell can obtain energy from reaction (3.6) and from reaction (3.8).[14]

$$CH_3COO^- + 4AQDS + 4H_2O \longrightarrow 2HCO_3^- + 4AH_2QDS + H^+ \quad (3.6)$$

$$4Pd^{2+} + 4H_2QDS \longrightarrow 4Pd^0 + 4AQDS + 8H^+ \quad (3.7)$$

$$CH_3COO^- + 4Pd^{2+} + 4H_2O \longrightarrow 4Pd^0 + 2HCO_3^- + 9H^+ \quad (3.8)$$

According to the experimental results and based on the information available concerning *G. sulfurreducens* physiology, two mechanisms for the reduction of palladium, in the presence and in the absence of AQDS, are demonstrated to occur (Figure 3.7).

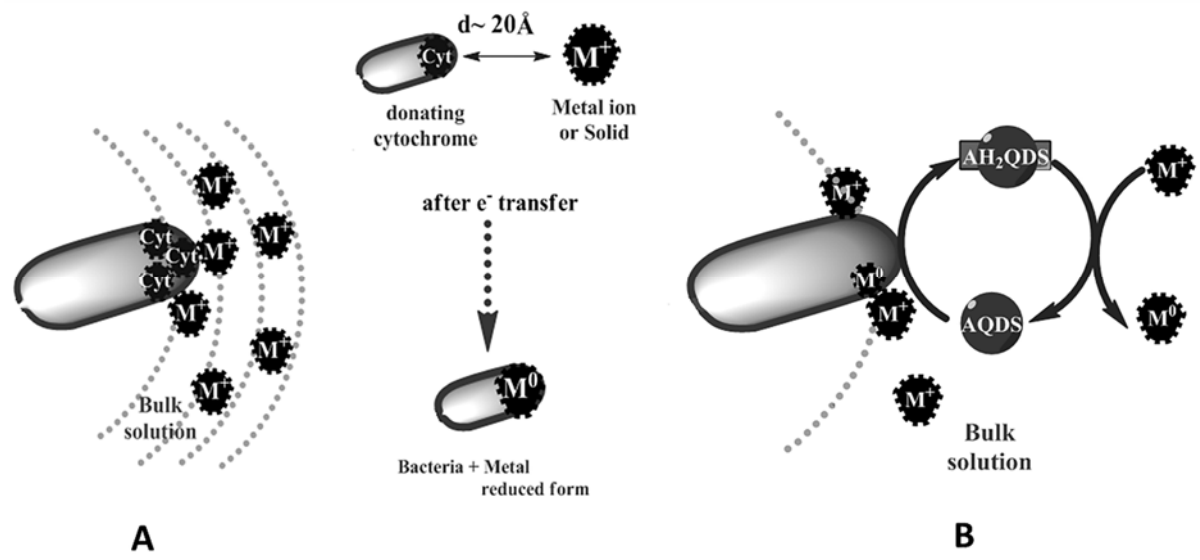

Figure 3.7 Mechanisms for palladium reduction by *Geobacter sulfurreducens:* directly through contact with outer membrane cytochromes (A) and indirectly using a redox mediator (B).

The mechanism for the reduction of soluble palladium species such as, $[Pd(NH_3)_4]^{2+}$, in the absence of redox mediator is represented in Figure 3.7A. The reduction of palladium species under this mechanism is conditioned to the direct contact of them with a reduced cytochrome (re-Cyt). The reaction products are Pd(0) and oxidized cytochrome (ox-Cyt), this direct contact-mechanism is shown in reaction (3.9). The distance between the metal species and the c-type cytochrome required for the electron transfer has been calculated to be ~20Å.[30]

$$Pd(II)Ligand + \quad re-Cyt \longrightarrow Pd(0) \quad + \quad ox-Cyt \qquad (3.9)$$

The specific steps involved in the reduction mechanism are explained and listed as follows:

1. Electrostatic interaction. According to speciation analysis, $[Pd(NH_3)_4]^{2+}$ is the predominant soluble palladium species and it is positively charged. *G. sulfurreducens* is a Gram-negative bacteria with a cell wall composed of 10-20% peptidoglycan, and an outer membrane, composed of lipoproteins, proteins, phospholipids and lipopolysaccharides, which confer to the cell an overall negative charge.[31,32] Thus, the first contact of the metal complex to the cell is based on coulombic interaction,[33] but the possibility of metal binding due to extracellular polysaccharides is not dismissed.[34]

2. Ligand substitution. After the first interaction of the metal with the cell wall, the possibility of the complex metal to interact with other characteristic ligands of the cell wall is likely to occur. FT-IR spectra obtained from cells of *G. sulfurreducens* after palladium reduction suggests that amide groups are involved in the reactions of palladium reduction. Namely, it has been proposed that nitrogen and oxygen atoms present in amide group (amino-N, carboxylate-O, amide-N and carbonyl-O) are potential ligands for binding transition-metal ions.[35,36] The amide group is neutral under the pH of the culture medium used, in the absence of metal ions, for that reason the terminal amino and carboxylate groups are expected as the principal sites for metal binding. Regarding peptides, it has been reported that palladium is able to promote deprotonation of nitrogen and to become coordinated with it.[37] Since Pd is classified as “soft acid” (according to Pearson’s HASB, Hard and Soft Acids and Bases principle) it indicates that Pd coordinates preferentially with ligands of decreasing electronegativity.[5] That explains the affinity of palladium for nitrogen bonding sites over oxygen bonding, considering the deprotonated amide nitrogen as a “soft base”.

The result of the interaction of palladium species $[Pd(NH_3)_4]^{2+}$ with the amide groups is subject to the presence of functional groups in the amino acids structure. In the

following lines it will be explained the possible interactions of Pd(II) with amide groups, associated to proteins from bacteria, according to the results obtained.

Figure 3.4 shows the interaction of amide group with palladium species via the amino nitrogen and carbonyl oxygen, structure (1), and a strong interaction occurs in structure (2) as a result of substitution of an amide nitrogen bound hydrogen resulting in a 5-membered ring (see Figure 3.4, structures 1 and 2).[36,38] According to Pettit and Bezer[38] and [3] with neutral tripeptides, Pd(II) promotes ionisations of one - NH_3^+ and two peptide nitrogens to give a planar complex at neutral pH with 3N and one carbonyl oxygen donor.

Figure 3.5 shows the interaction of palladium species with a sulfur containing amino acid. This example illustrates that Pd(II) first interacts with sulfur atom (deprotonated SH group) (see structure 1 of Figure 3.5) and in a next step, Pd(II) is bound through the amino group.[40] Thus this interaction gives a 5-membered ring (structure 2 of Figure 3.5).

Besides the interaction previously described and considering that the outer membrane *of G. sulfurreducens* is mainly composed by lipopolysaccharides and phospholipids,[32] metal binding through phosphoryl group may take place, as shown in reaction (3.10).[41,42] The latter was also inferred from FT-IR analysis (Figure 3A), since results showed that intensity of phosphoryl vibrations changed when comparing cells samples before and after being exposed to palladium reduction.

$$\left[Pd\left(NH_3\right)_4\right]^{2+} + R-POH \longrightarrow R-PO\left[Pd\left(NH_3\right)_4\right]^{+} + \mathrm{NH_4^+} \qquad (3.10)$$

3. *Reduction and nucleation*. Once the palladium complex has interacted with the membrane cell, which means that the distance required of ~20Å has been overcome, the reduction step takes place and re-Cyt delivers the electrons to become ox-Cyt. After the binding of the metal complex to specific sites has occurred, nucleation sites will also be available and micro-precipitation might occur.[41]

The exact mechanism of extracellular respiration is not known, but many reports have postulated that electron transfer proceeds through a series of multiheme cytochromes with overlapping midpoint potentials from the inner membrane through

the periplasm and the outer membrane.[20] The possible pathway for electron transfer suggested for Fe(III) reduction[20] adapted to the present case is shown in Figure 3.8. Graphical pathway from cytoplasm, periplasm and outer membrane is present in Figure 3.8A. The pathway according to reduction potentials considering the three palladium predominant species $Pd(NH_3)_4^{2+}$ (-0.024 V) , $Pd(NH_3)_3^{2+}$ (-0.214 V) and $Pd(OH_2)_{(s)}$ (+0.07 V) is represented in Figure 3.8B.

The mechanism illustrated in Figure 3.7B, when AQDS is present, considers that bacteria reduce AQDS to AH_2QDS, which abiotically reduce Pd(II) to Pd(0) with the concomitant re-oxidation of AH_2QDS, and this process continues as long as bacteria continue reducing the redox mediator. It has previously been documented that AQDS-mediated reduction of Pd(II) promotes extracellular deposition of palladium NP's[8] and under these conditions palladium reduction can occur by both mechanisms explained. The former statement was also proved by zeta potential measurements; the value of -39.82 ± 6.32 mV for Pd NP's is similar to the value reported by Sathishkumar[15] and Xu. [43]

Extracellular formation of Pd(0) NP's might potentially represent an advantage for the application of simple methods of separation such as ultracentrifugation.[44] Moreover, extracellular Pd NP's could have better catalytic properties because the possibility of bindings between sulfur compounds (thiol groups of proteins from bacteria) and Pd(0) is avoided, hence the possibility of catalyst poisoning is prevented.[13, 45,46]

The present work contributes to a better understanding of the process concerning Pd(II) reduction by bacteria. Furthermore, the proposed models well describe both mechanisms, considering important elements such as speciation, delay factor, area of bacteria and mass transfer considerations for direct and quinone-mediated microbial reduction of Pd(II). The obtained parameters also give information about the process impact on the cells and the influence of external factors on direct and quinone-mediated palladium reduction. This information could promote industrial scaling of microbial processes for metal recovery from metal-containing effluents.

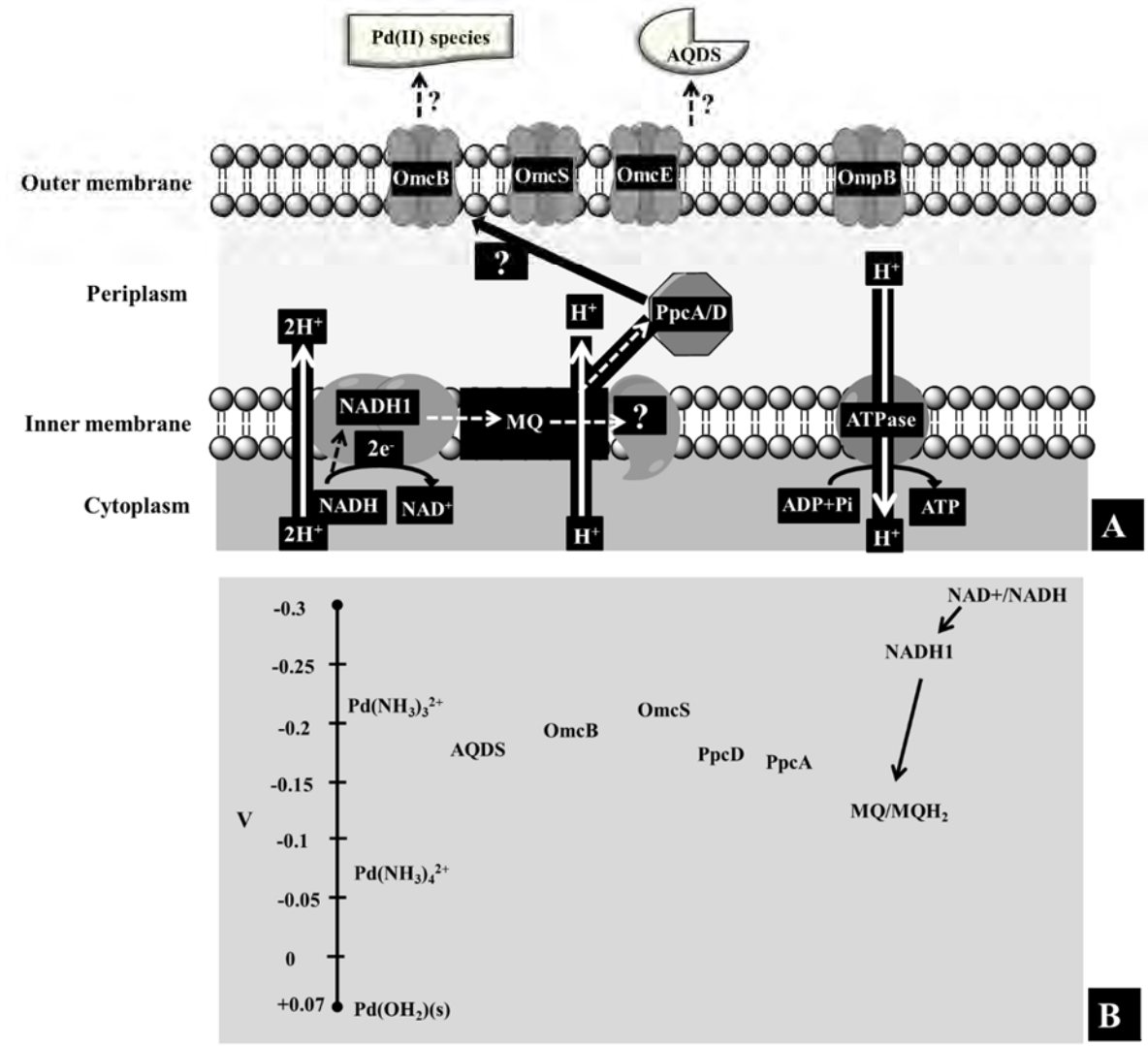

Figure 3.8 Model of *G. sulfurreducens* suggested pathway for Pd(II) reduction (adapted and modified from Bird et al., 2011): (A) Electron transport through periplasm and membranes and (B) Reduction potentials involved in the electron transfer pathway. Where: PpcA, PpcD, OmcB. OmcE and OmcS are cytochrome c-type proteins; OmpB, multicopper protein; NADH1, NADH dehydrogenase; MQ, menaquinone.

3.6 References

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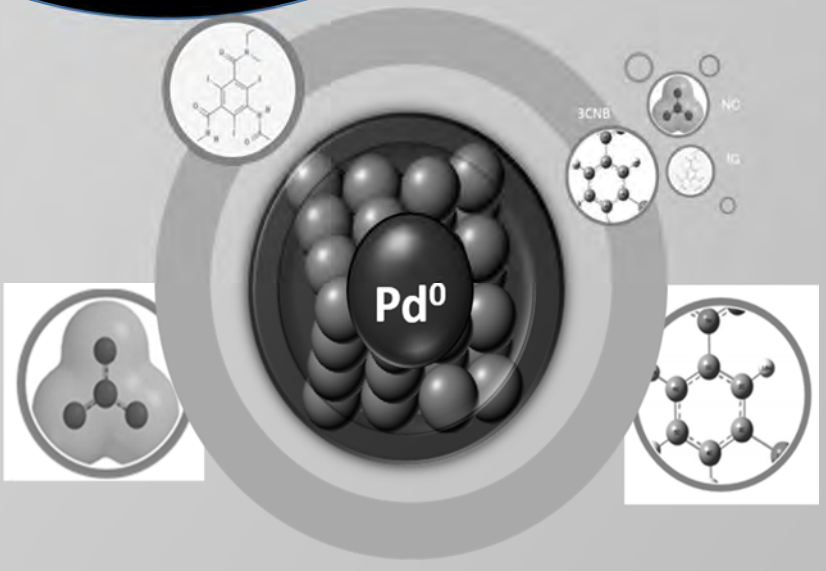
CHAPTER 4

Recovery of palladium (II) by methanogenic granular sludge



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4.1 Abstract

This is the first report that demonstrates the ability of methanogenic granular sludge to reduce Pd(II) to Pd(0). Different electron donors were evaluated for their effectiveness in promoting Pd reduction. Formate and H_2 fostered both chemically and biologically mediated Pd reduction. Ethanol only promoted the reduction of Pd(II) under biotic conditions and the reduction was likely mediated by H_2 released from ethanol fermentation. No reduction was observed in biotic or abiotic assays with all other substrates tested (acetate, lactate and pyruvate) although a large fraction of the total Pd was removed from the liquid medium likely due to biosorption. Pd(II) displayed severe inhibition towards acetoclastic and hydrogenotrophic methanogens, as indicated by 50% inhibiting concentrations as low as 0.96 and 2.7 mg L□□, respectively. The results obtained indicate the potential of utilizing anaerobic granular sludge bioreactor technology as a practical and promising option for Pd(II) reduction and recovery offering advantages over pure cultures.

4.2 Introduction

The increasing demand for platinum group metals, raise in price and limited and finite ore sources have motivated the searching of new techniques and methods to achieve their efficient recovery and recycling.[1,2] Particularly, palladium (Pd) is one of the most demanded metals in chemical synthesis processes and in automobile catalytic converters.[3] For that reason many efforts have been focused on studying alternatives to reclaim this metal from wastes and to regenerate Pd(0) by reduction of soluble Pd(II).[2,4]

Traditional methods of recycling, such as hydro- and pyrometallurgical processes, imply important costs and time as well as the generation of a secondary waste streams containing the chemicals used in the process.[5] Biological processes have emerged as alternative for metals recovery. Biological-mediated reduction and recovery of metals is attractive because it involves less aggressive conditions, fairly easy manipulation of the process and avoid the production of toxic byproducts.[6]

Several studies have reported on the use of pure microbial cultures for achieving Pd(II) reduction for the recovery of Pd(0) as high value nanoparticles. *Shewanella oneidensis, Desulfovibrio desulfuricans and Geobacter sufurreducens* are among the most studied microorganisms capable of Pd(II) reduction.[7–12] The process mainly occurs under anaerobic conditions and it is mediated by a broad spectrum of Gram-negative bacteria using formate and hydrogen as typical propitious electron donors. Reduction of Pd(II) by a sulfate reducing bcateria (SRB) enrichment[13] and by an aerobic mixed culture maintained under fermentative conditions [14] has also been demonstrated in very recent studies.[3]

However, until now methanogenic consortia have not been explored for palladium reduction. Furthermore, there are no studies reporting on the inhibitory effect of this heavy metal (Pd) on methanogenesis.

The use of mixed microbial consortia to reduce and recover palladium would favor the applicability of biological recovery systems since design and operation would be more feasible. Moreover, utilization of mixed cultures can offer significant advantages compared to pure cultures such as ability to adapt to minor changes in condition, beneficial effects of synergistic interactions among members of the association, and the feasibility of operating under non aseptic conditions, facilitating process control and reducing maintenance and operation costs.[15–17]

Hence, the aim of this investigation was to study two main aspects that had not been previously reported: (i) the capacity of anaerobic granular sludge to reduce Pd(II) to Pd(0) using different electron donating substrates and (ii) the inhibitory effect of Pd(II) on the acetoclastic and hydrogenotrophic methanogenic activity.

4.3 Materials and methods

4.3.1 Source of anaerobic granular sludge

The anaerobic granular sludge was obtained from a full-scale upflow anaerobic sludge blanket (UASB) reactor treating brewery wastewater (Mahou, Guadalajara,

Spain). The content of volatile suspended solids (VSS) was 4.77% of the wet weight. The anaerobic sludge was stored at 4°C under a N_2 atmosphere before use.

4.3.2 Batch palladium reduction experiments

The experiments were performed using a basal medium containing (g L^{-1}): $NaHCO_3$ (2.5), NH_4Cl (0.25), $NaH_2PO_4 \cdot H_2O$ (0.006), and KCl (0.1). All assays were carried out in 160-mL glass serum bottles capped with inert stoppers with 50 mL of total liquid volume of anaerobic basal medium. The anaerobic granular sludge was added to yield a concentration of 0.5 g VSS L^{-1}. Subsequently liquid and headspace was flushed with N_2/CO_2 (80:20, v/v) to preclude the presence of atmospheric oxygen. Depending on the experimental set-up, an appropriate volume of ethanol, formate, lactate, pyruvate or acetate stock solutions was added to give a final concentration of 0.115 g chemical oxygen demand (COD) L^{-1}.

In bioassays utilizing H_2 as electron donor, H_2 was supplied as a gas mixture of H_2/CO_2 (80:20 v/v) by direct injection to give a final concentration of 2.4 mM (based on the volume of the liquid medium). This value corresponds to the stoichiometric yield of H_2 expected from ethanol fermentation at the concentration added in the assay described above.

Finally, Pd(II) was provided to each bottle in the form of Na_2PdCl_4 to give a final concentration of 25 mg of Pd(II) L^{-1}. Control experiments were carried out in cell-free media. All assays were incubated at 30°C at 110 ± 10 rpm. The assays were performed in duplicate and samples of 1 mL were analyzed at specific time intervals to monitor the change in the Pd and electron donor concentrations.

4.3.3 Batch inhibitory bioassays for acetoclastic and hydrogenotrophic methanogenic activity

Batch experiments were conducted in duplicate using the same anaerobic basal medium described for the Pd reduction experiments. Glass serum bottles (160 mL) were amended with 25 mL of medium, microbial inoculum (0.5 g VSS L^{-1}) and the required volume of Na_2PdCl_4 stock solution to give different concentrations of Pd(II)

according to the experimental set-up (0.05, 0.1, 1.0, 2.0, 3.1, 12.5, 25.0 and 30.0 mg of Pd(II) L^{-1} in each bottle). All bottles were flushed following the procedure previously described for the batch experiments in order to ensure anaerobic conditions. Control experiments without added Pd(II) were included in order to assess the inhibition of methanogenic activity. Sodium acetate (2 g COD L^{-1}) or hydrogen gas were added as electron donor. H_2 was supplied to a final headspace pressure of 0.5 atm of H_2/CO_2 applied as an overpressure after first flushing the assay bottles with the N_2/CO_2 gas mixture. All the assays were incubated at 30 ± 2°C at 110 rpm. A gas sample of 100 µL was withdrawn daily from each bottle and methane production was analyzed until the maximum theoretical methane production was reached.

In order to test the inhibitory impact of Pd(II) on hydrogenotrophic methanogenic activity, the anaerobic sludge was pre-exposed to Pd(II) in the absence of exogenous electron donor in order to avoid interference because of fast palladium reduction. These assays consisted of 60 h of pre-incubation at the corresponding Pd(II) concentration followed by the addition of H_2 as previously described.

The normalized methanogenic activity was calculated as the percentage of the ratio of the maximum methane production rates in treatments for the different Pd(II) concentrations and the control (without added palladium). The initial concentration of Pd(II) causing 50% inhibition was referred to as IC_{50}. This value was calculated by interpolation of graphs plotting the normalized activity as a function of the Pd(II) concentration.

4.3.4 Sorption isotherms

Sorption isotherm experiments with anaerobic granular sludge were conducted in duplicate using the same anaerobic basal medium described for the Pd reduction experiments. Glass serum bottles (160 mL) were amended with an appropriate volume of medium and microbial inoculum (0.5 g VSS L^{-1}). The solution volume was 50 mL and Pd(II) was added from a Na_2PdCl_4 stock solution to give the final concentration required (0.1, 0.5, 2.5, 5, 15, 25, 30, 35, 50, 75 and 100 mg of Pd(II) L^{-1} in each bottle). Control flasks lacking anaerobic granular sludge were run in

parallel to correct for possible removal of palladium by other mechanisms than adsorption. Flasks were shaken at 110 rpm for 48 h at 30°C. At the end of the incubation, liquid samples from all the flasks were obtained for analysis of dissolved palladium.

Langmuir and Freundlich models were used to fit the experimental data. The Langmuir isotherm is defined by $C_s = \frac{a*b*C_e}{a+b*C_e}$ where Cs is the concentration of the solute in the solid phase (expressed as mg Pd g^{-1} VSS), Ce is the equilibrium concentration of the solute in solution (expressed as mg Pd L^{-1}), *a* and *b* are Langmuir adsorption constants; *a* represents the maximum achievable surface concentration of the solute, and *b* is the equilibrium constant for the sorption reaction. The Langmuir equation assumes that there is no interaction between the sorbate molecules and that the sorption is localized in a monolayer. The Freundlich equation is widely used and has been found to describe adequately the adsorption process for many compounds in dilute solution, it is defined by $C_s = K_F\, C_e^n$ where K_F is the Freundlich adsorption constant or capacity factor and *n* is the Freundlich exponent which provides a measure for the sorption intensity.

4.3.5 Analytical techniques

The Pd content in liquid samples was analyzed by inductively coupled plasma-optical emission spectroscopy (ICP-OES Optima 2100 DV, Perkin Elmer, Waltham, MA, USA) at a wavelength 340.5 nm after filtering by 0.45 µm Millipore membrane filters (Billerica, MA, USA). The detection limit for Pd was 10 µg L^{-1}.

The concentrations of ethanol and acetate were measured by gas chromatography (7890A GC System, Agilent Technologies, Santa Clara, CA, USA) using a fused silica Stabilwax®-DA column (30 m length, 0.5 mm ID, Restek, State College, PA, USA) and a flame ionization detector. The temperature of the injector port and the detector were set at 280°C. The initial temperature of the column was 100°C and it was increased up to 150°C at a rate of 8°C per min. Helium was used as the carrier gas (5.2 mL min^{-1}), and air and hydrogen as flame source. The injection volume was 1 µL.

Methane content of headspace samples was analyzed by gas chromatography (GC) using a Hewlett–Packard 5890 Series II system (Agilent Technologies, Palo Alto, CA) using a Restek Stabilwax-DA fused silica capillary column (30 m length, 0.53 mm ID, Restek Corporation, Bellefonte, PA) and a flame ionization detector. The temperature of the column was 140°C and the injector and detector temperatures were 180 and 250°C, respectively. Helium was used as the carrier gas at a flow rate of 18 mL min^{-1} with a split flow of 85 mL min^{-1}.

4.3.6 X-ray diffraction analysis

Analysis of the products derived from different Pd(II) reduction experiments was carried out in an X-ray powder diffractometer (Scintag's XDS 2000) wavelength of 1.5406 A Cu-Kα1; generator settings of 40 kV and 40 mA; continuous scan of 2.0 deg/min from 10° to 70° 2θ. Samples for this analysis were prepared as described previously by Lloyd et al. (1998). XRD patterns were indexed to ICDD card 05-0681 (fcc palladium syn).

4.3.7 Scanning electron microscopy

Samples from different assays were prepared for scanning electron microscopy (SEM) by fixation in 2.5% glutaraldehyde for 30 min, washed with 2 changes in 0.1 M PIPES buffer (pH 7.4) and further fixed in 2% osmium tetroxide for 15 min. Pellets were washed three times in deionized water and dehydrated through a graded ethanol series (50, 70, 90 and 100%) immersed in hexamethyldisilazane (HMDS) for 5 min, and air dried.

Samples were coated with gold. The images were collected on an FEI Inspect-S SEM (FEI Europe, Eindhoven, The Netherlands) with a Thermo Noran System Six Energy Dispersive Spectrometer (EDS) system. SEM images were collected at 30 kV.

4.3.8 Chemicals

Palladium source was purchased from Alfa Aesar as $Cl_4Na_2Pd*3H_2O$ (99% purity). Sodium acetate (99.9%) was purchased from Sigma–Aldrich (St. Louis, MO, USA).

H_2/CO_2 (80/20, v/v) gas mix was delivered by Airweld (Phoenix, AZ, USA). N_2/CO_2 (80/20, v/v) gas mix and CH_4 standard gas (99%) were acquired from Air Liquid America (Plumstedsville, PA, USA). Sodium bicarbonate was purchased from Fisher Scientific (Pittsburgh, PA, USA).

4.4 Results and discussion

4.4.1 Pd(II)removal by anaerobic granular sludge

The removal of Pd (II) by anaerobic consortium was evaluated using different electron donors, *i.e*., ethanol, formate, hydrogen, acetate, lactate and pyruvate. Results obtained showed that Pd was largely removed in inoculated assays supplied with the different exogenous substrates (Figures 4.1 and 4.2, Table 4.1). Maximum rates of Pd(II) removal were calculated for all of the treatments and are listed on Table 4.2. In assays in which H_2 or formate were used as electron donors, removal of Pd(II) was observed both in inoculated and cell-free control assays. The presence of microbial cells increased the rate of Pd(II) removal substantially (1.3-1.4-fold) compared to the cell-free controls (Table 4.2).

In contrast with the assays spiked with formate and H_2, removal of Pd(II) in assays supplemented with acetate, pyruvate, lactate, or ethanol as electron donors was only observed in the inoculated treatments (Figure 4.1B-4.1E). The results obtained with the latter four organic compounds indicate that the decrease in the Pd concentration required the presence of microbial biomass and demonstrate the ineffectiveness of the four chemicals to catalyze directly the abiotic reduction of this metal ion.

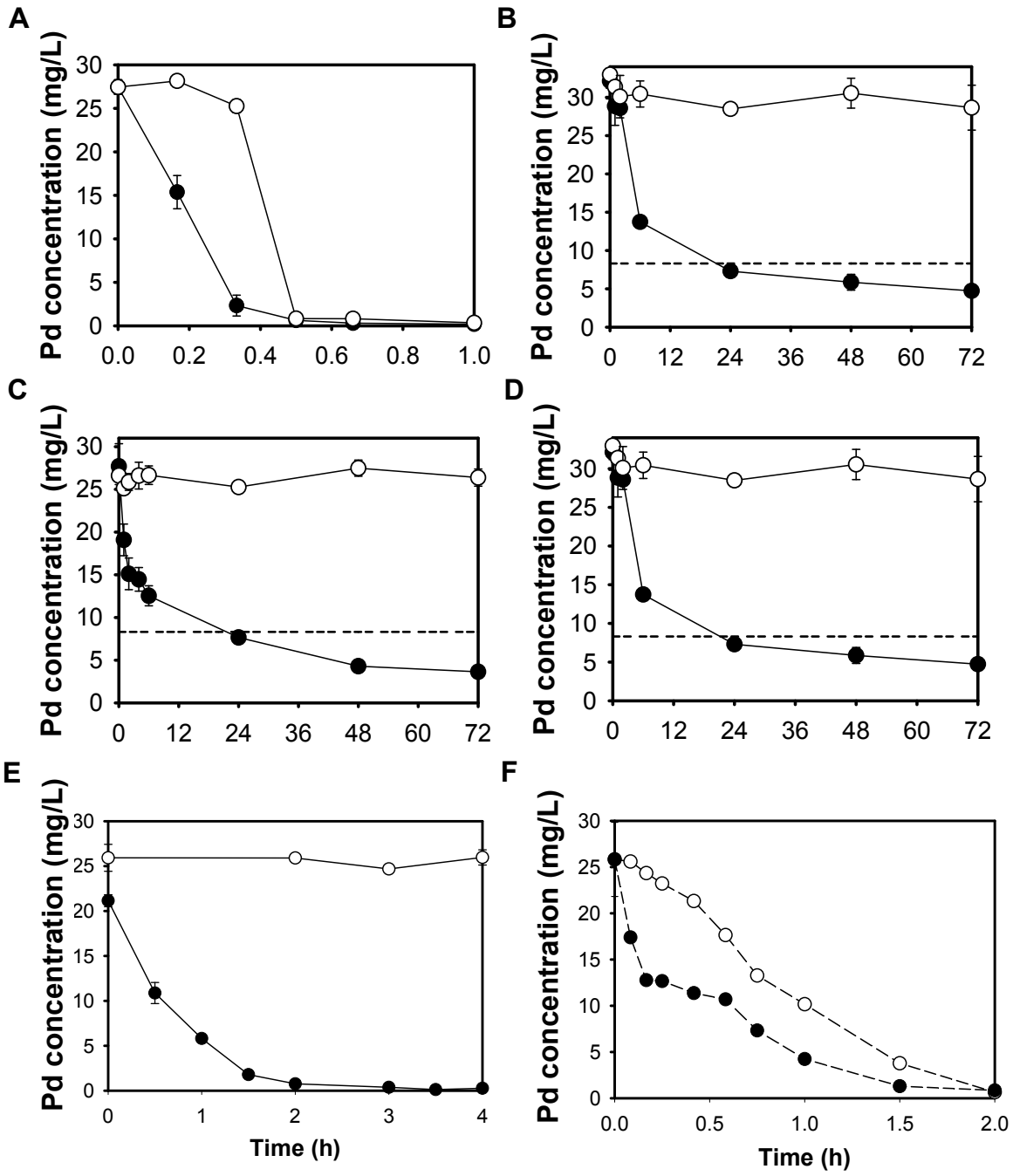

Figure 4.1 Kinetic evolution of palladium removal by anaerobic granular sludge in inoculated- (●) and abiotic assays (○) supplemented with formate (A), acetate (B), pyruvate (C), lactate (D), ethanol (E), and hydrogen (F). The trace line (−−− −) indicates the concentration of soluble palladium expected to remain in the liquid phase (8 mg Pd L^{-1}) after sorption of the estimate amount of palladium calculated from the results of sorption isotherms (1.62 mg Pd g^{-1} VSS) for an initial Pd(II) concentration of 25 mg L^{-1}.

Table 4.1 Removal of palladium by anaerobic granular sludge supplied with different electron donors after 6 to 24 h of incubation.

Electron donor	Final Pd concentration in solution (mg L^{-1})	Pd removal (%)
Ethanol	0.07 ± 0.95	99.73 ± 0.16
Formate	0.19 ± 0.04	99.26 ± 1.20
Hydrogen	0.03 ± 0.31	99.88 ± 0.23
Acetate	3.34 ± 0.06	87.07 ± 3.68
Lactate	4.73 ± 0.13	81.69 ± 0.50
Pyruvate	3.64 ± 0.05	85.91 ± 0.19

Table 4.2 Maximum rates of palladium removal determined in bioassays and cell-free controls supplied with different electron donors

Electron donor	Removal rate Biological assay (mg Pd $(L \cdot h)^{-1}$)	Removal rate cell-free control (mg Pd $(L \cdot h)^{-1}$)
Ethanol	19.38 ± 0.95	-
Formate	75.43 ± 1.02	53.22 ± 0.54
Hydrogen	22.26 ± 1.35	17.00 ± 0.73
Acetate	6.86 ± 0.54	-
Lactate	3.08 ± 1.43	-
Pyruvate	6.29 ± 2.12	-

The maximum Pd(II) removal rate determined in the assays with acetate, lactate, pyruvate ranged from 3.1 to 6.9 mg Pd $(L \cdot h)^{-1}$, values which are considerably lower compared to those determined in experiments spiked with other electron donors (Table 4.2). A higher Pd(II) removal rate was observed with ethanol (19.4 mg Pd $(L \cdot h)^{-1}$). Palladium reduction with ethanol was fast and 99.7% of the soluble Pd(II) was removed after only two hours of incubation (Table 4.1).

Physical changes were observed in the color of the medium and/or sludge in some of the assays performed. In the case of inoculated experiments in which formate, ethanol and hydrogen were used as exogenous electron donor a change in the color of the granules, which turned black, was evident shortly after the electron donor was added. Black precipitates were also observed in the medium of abiotic controls amended with hydrogen and formate. In contrast color in the medium of experiments amended with the other electron donors (acetate, lactate and pyruvate) changed from brown to light yellow and biomass turned dark brown likely because of biosorption.

Experiments performed in an attempt to associate the theoretical H_2 produced by the amount of ethanol added resulted in similar kinetic behavior compared with ethanol assays as can be seen in Figures 1E and 1F. Interestingly, the rates of Pd(II) removal in inoculated assays with ethanol and H_2 were relatively similar, 19.4 and 22.3mg Pd $(L \cdot h)^{-1}$, respectively.

Results obtained from experiments supplemented with two consecutives spikes of Pd(II) and ethanol (at time 0 h and 24 h) are shown in Figure 4.2. This experiment revealed four main findings: (i) anaerobic granular sludge is able to reduce two consecutive feedings of Pd(II), (25 mg Pd(II) L^{-1} at a time, total of 50 mg Pd(II) L^{-1}), (ii) the ethanol consumption rate in the second feeding is slower than in the first period of incubation, (iii) acetate produced is accumulated in the system, and (iv) methane production is increasingly inhibited with time (Figure 4.2A). As result of these observations, after 65 h of incubation the balance of total electron equivalents is represented in a higher percentage by acetate and in a lesser extent by methane produced while the fraction of electron equivalents required for Pd reduction is less than 5% (Figure 4.2B).

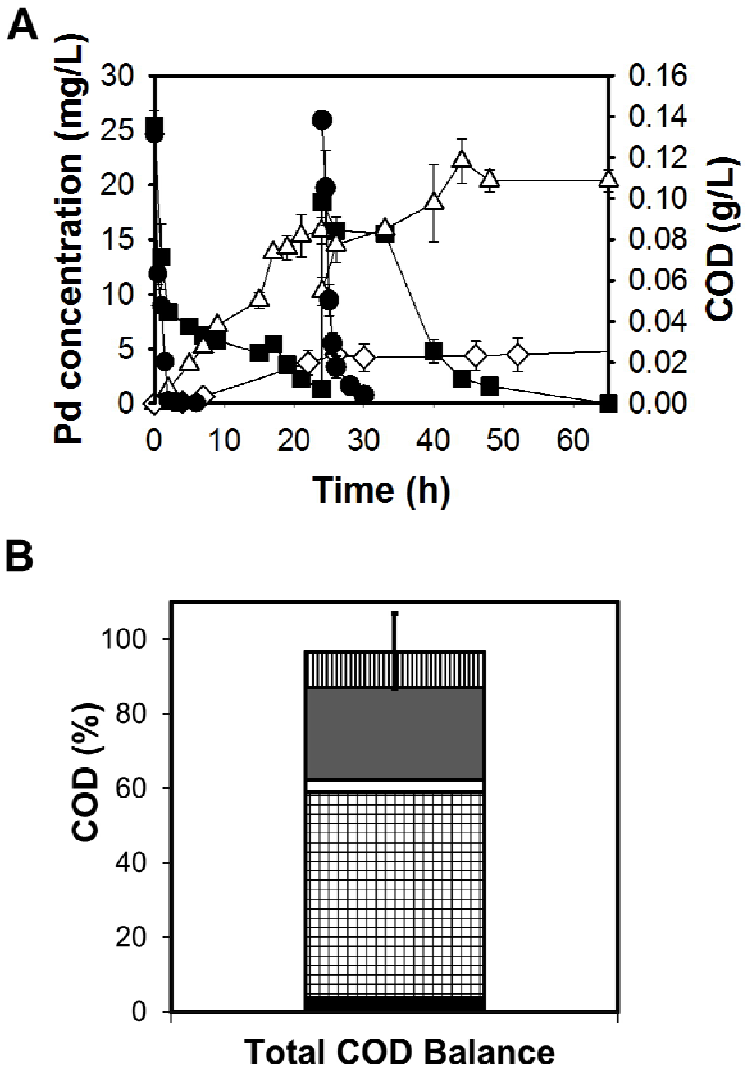

Figure 4.2 (A) Kinetic evolution of palladium removal and ethanol biotransformation. Dissolved palladium (●); acetate (◊); ethanol (■), and methane (∆). (B) Final COD balance represented by cells (vertical lines fill), methane (grey fill), Pd reduced (white), acetate (small grid) and ethanol (black fill).

4.4.2 Pd(II) removal by biosorption onto anaerobic granular sludge

In order to investigate the influence of biosorption onto the anaerobic granular sludge on Pd(II) removal, sorption isotherm experiments were carried using different Pd(II) concentrations (Figure 4.3).

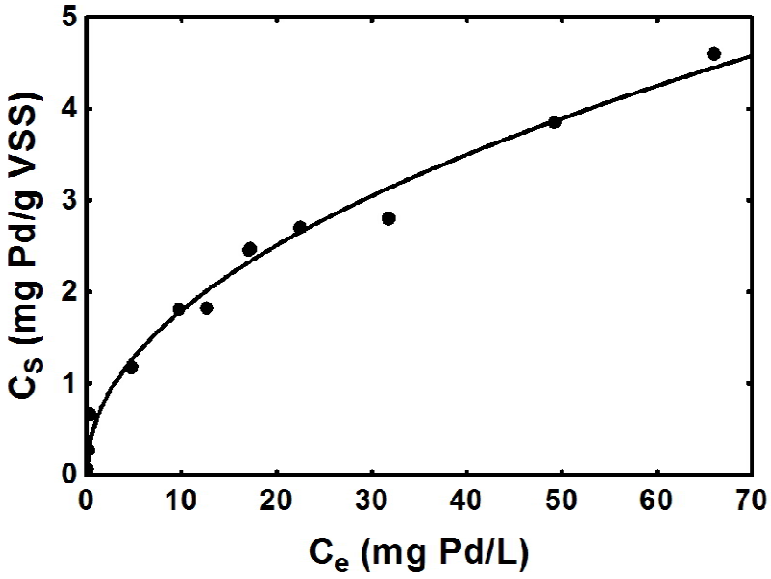

Figure 4.3 Biosorption isotherms of Pd(II) onto anaerobic granular sludge (●) determined at 30°C. Experimental data fit to Freundlich model (solid line). Error bars (shown if larger that the symbols) represent standard deviations of duplicate assays.

Freundlich and Langmuir adsorption isotherms were used to model the equilibrium adsorption data obtained (Figure 4.4). The equilibrium adsorption data showed a good fit to the Freundlich and Langmuir models, as evidenced by the high R^2 values (0.96-0.98) obtained for both models. The isotherm parameters (Table 4.3) demonstrate that Pd(II) has a moderate affinity for the biomass and indicate that a fraction of the Pd removed in the inoculated experiments was due to biosorption. At the initial concentration of Pd(II) added in the bioassays (approx. 25 mg L^{-1}), the equilibrium concentration of Pd(II) adsorbed onto the sludge is 1.6 mg Pd g^{-1} VSS. The dotted lines in Figure 4.1 shows the concentration of soluble palladium that is expected to remain in the liquid phase after sorption of the amount of palladium calculated from the sorption isotherms.

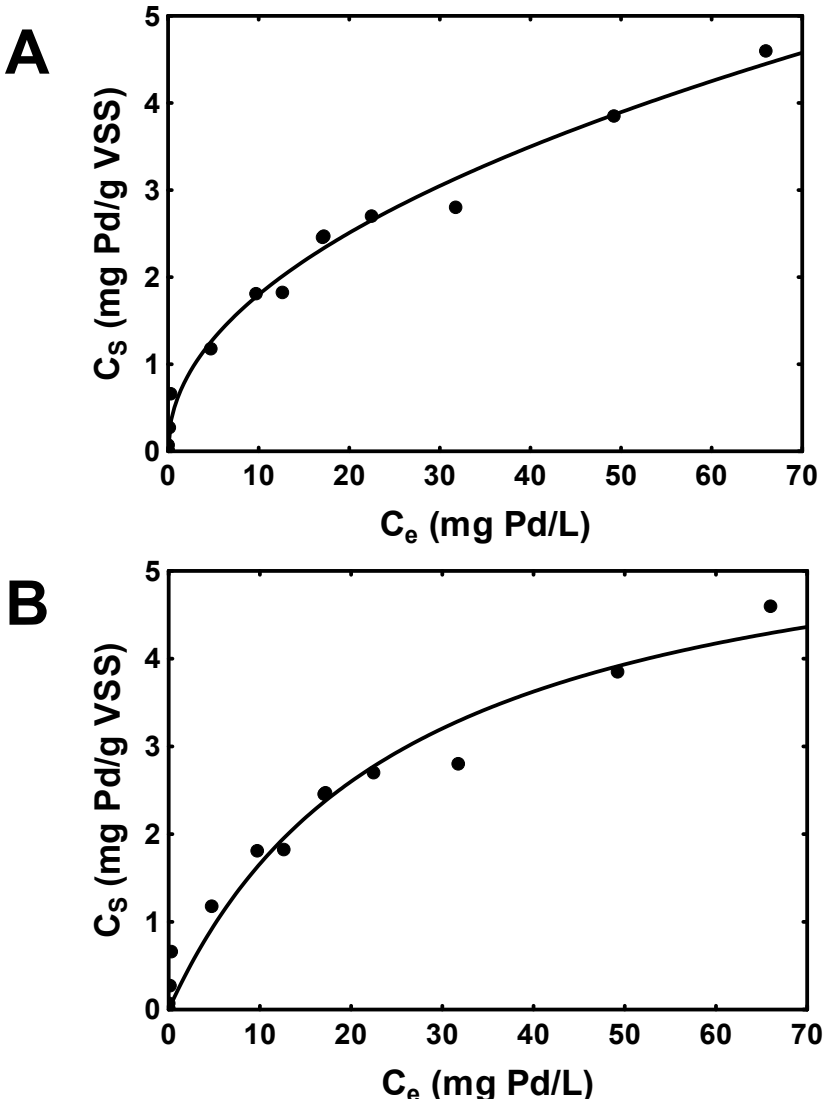

Figure 4.4 Biosorption isotherms of Pd(II) onto anaerobic granular sludge (●) determined at 30°C. Experimental data fit to (solid line) (A) Freundlich and (b) Langmuir model.

Although biosorption mechanisms are poorly understood, some studies suggest that metals are bound to sludge solids by chemisorption through weakly acidic organic functional groups[18,19], association by organometallic complexation, and binding induced by extracellular ligands[19]. Several studies have demonstrated that Pd(II) can adsorb to the cell surface by interacting with organic functional groups such as amine, amide, carboxyl and phosphoryl groups.[20–23]

Table 4.3 Langmuir isotherm constants *a* (mg Pd g^{-1} VSS) and *b* (l mg^{-1} Pd) and Freundlich isotherm constants K_F [(mg Pd g^{-1} VSS)(mg Pd $L^{-1})^{-v}$] and *n* for the adsorption of Pd onto methanogenic anaerobic granular sludge at 30°C and solution pH 7.

Langmuir isotherm			Freundlich isotherm		
a	*b*	r^2	K_F	*n*	r^2
0.038	5.98	0.96	0.59	0.48	0.98

4.4.3 Characterization of Pd associated to the anaerobic sludge

The product obtained from the different experiments was analyzed by XRD and the results confirmed the formation of Pd(0) with ethanol, hydrogen and formate used as electron donors (Figure 4.5). In XRD patterns of samples from experiments performed with formate, hydrogen and ethanol it was found characteristic diffraction peaks of crystalline Pd(0) particles at 2θ values of 40°, 47°, and 68° in all of the three samples (Figure 4.5A). In contrast with these results, XRD patterns of samples from experiments spiked with acetate, lactate and pyruvate resulted in amorphous products with a hint of Pd $(PO_3)_2$ (ICDD 45-0165) (Figure 4.5B).

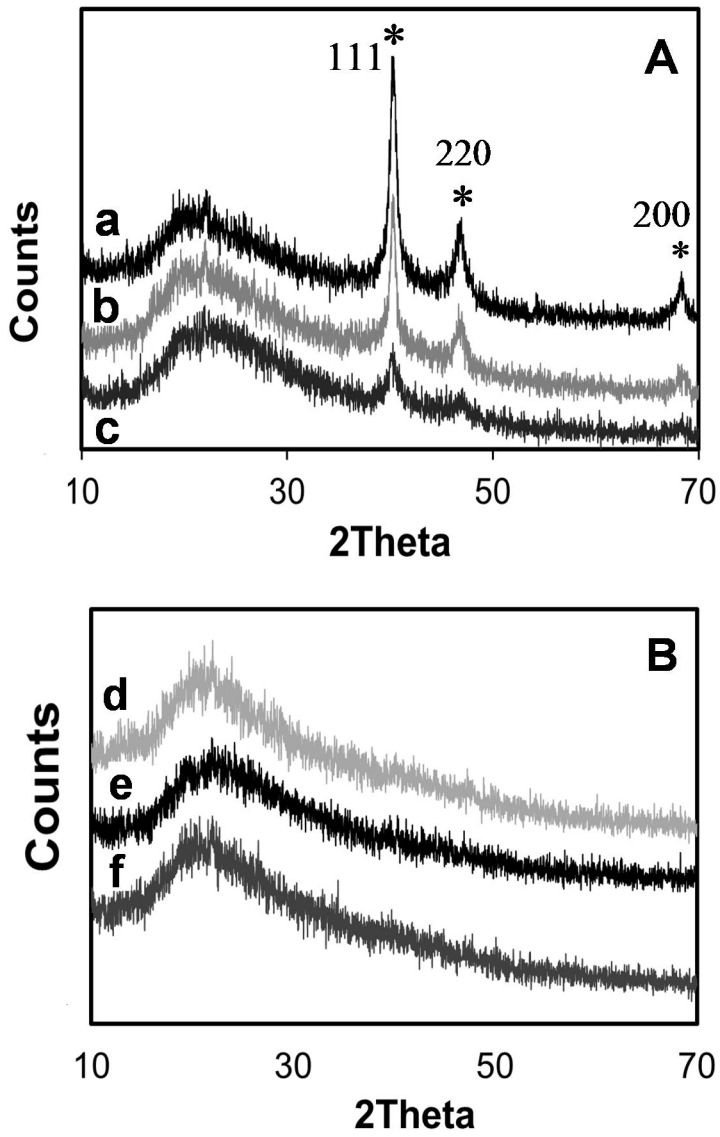

Figure 4.5 (A) Comparison of XRD patterns corresponding to cells and black precipitates obtained in experiments supplemented with ethanol (a), formate (b), hydrogen (c), pyruvate (d), lactate (e), and acetate (f).

Samples of the granules from experiments in which the formation of Pd(0) was confirmed were also analyzed by SEM-EDS and micrographs showed aggregates rich in Pd (Figure 4.6). Figure 4.6B shows Pd-rich aggregates in biomass samples obtained from bioassays spiked with ethanol. In assays with ethanol, palladium reduction is likely mediated by the H_2 released from ethanol fermentation and

therefore, there is a high level of Pd(0) association with the microbial cells. In assays supplemented with H_2, the rapid Pd(II) reduction is chemically mediated and occurs mainly in the bulk solution, leading to the formation of dense aggregates that have a random distribution and are not tightly associated with microbial cells as can be observed in Figure 4.6C.

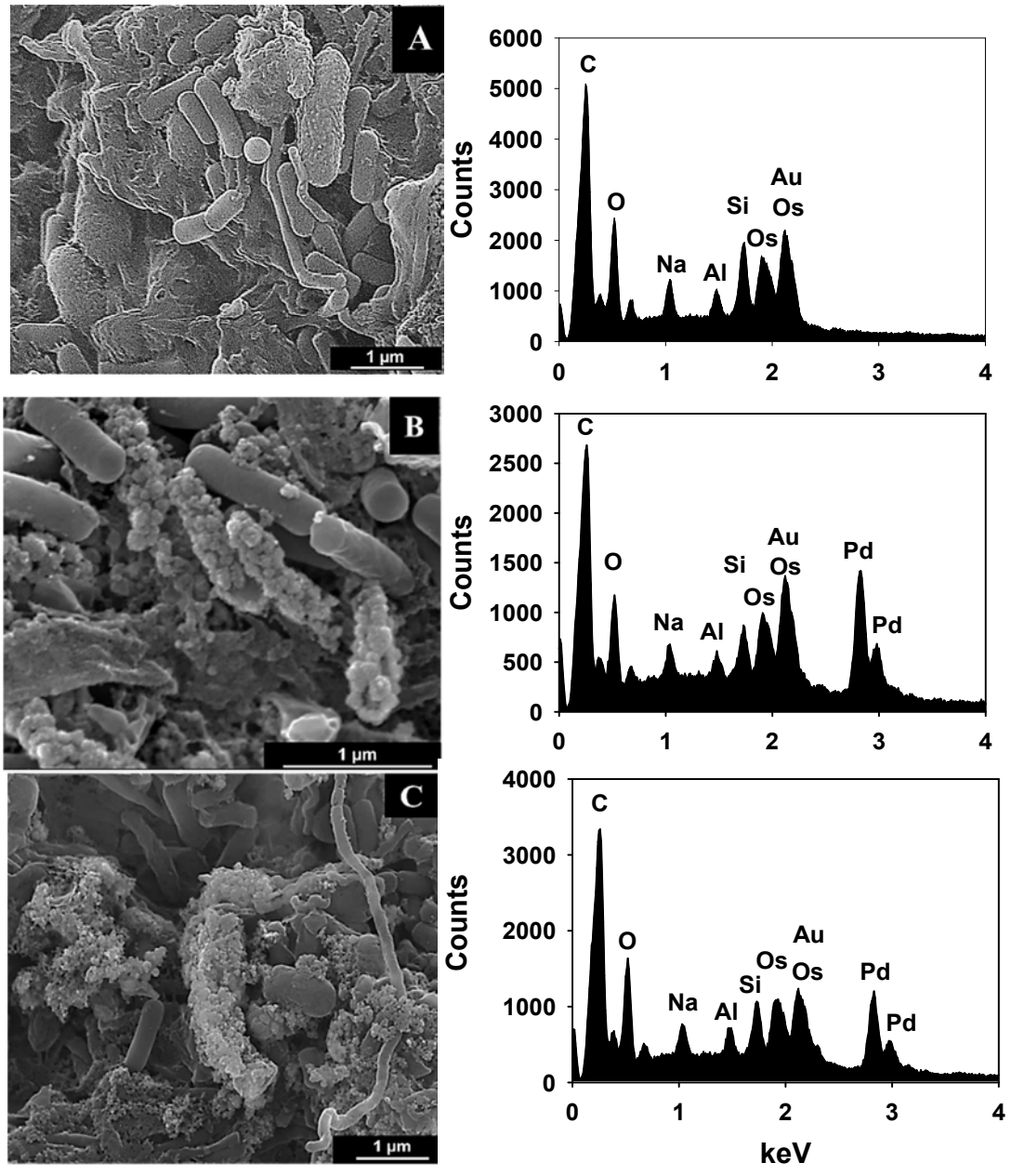

Figure 4.6 SEM images and EDS analysis of anaerobic granular sludge and Pd(0) in the inoculum used in the bioassays (A) and the anaerobic sludge obtained from incubations supplemented with acetate (B), and hydrogen (C) as electron donors.

The later mentioned is expected since a typical UASB granule exhibits a complex layer structure with few craterous pores, an outer layer composed of various types of bacteria with different shapes (filaments, rods, cocci) and strands of extracellular polymer[24,25] and, therefore, sites available for Pd(0) nucleation are heterogeneously distributed.

4.4.4 Mechanisms of Pd(II) removal by methanogenic granular sludge

The results obtained indicate that removal of Pd(II) in the bioassays inoculated with anaerobic granular sludge can be governed by two different mechanisms, reduction to insoluble Pd(0) and adsorption onto the microbial biomass. Whereas reduction was the main Pd removal mechanism in bioassays spiked with formate, hydrogen and ethanol, biosorption appears to be the dominant mechanism in bioassays with acetate, lactate and pyruvate. Precipitation is not expected to play a major role in the removal of Pd under the conditions of these experiments as indicated by the negligible losses of Pd in abiotic assays where chemical reaction did not occur (Figures 4.1B-4.1E).

Palladium biosorption, without Pd reduction, was found to occur in experiments performed with acetate, pyruvate and lactate as electron donors in which a decrease in the total Pd concentration was observed but this partial loss was not associated with Pd reduction as confirmed by the absence of the patterns typical of Pd(0) in the XRD spectra of the samples (Figure 4.5). The rapid removal of Pd(II) observed during the initial 4 hours followed by a much slower decrease thereafter (Figures 4.1A-4.1C) suggest rapid adsorption of approximately 52% of the Pd added, which is consistent with the results of the adsorption isotherms.

Several studies have investigated the reduction of Pd(II) by means of pure cultures but little is known about the reduction of Pd(II) by microbial consortia. There are few studies reporting on the reduction of related metals such as Rh(III) and Pt(IV) by mixed consortia of SRB[17,26] and a recent report describing the reduction of Pd(II) by a SRB enrichment culture[13]. The results obtained in this study demonstrate that anaerobic granular sludge is able to reduce Pd(II) to Pd(0) when an appropriate

electron donor is available and it constitutes the first report on the reduction of this metal by a methanogenic consortium.

This study demonstrated that formate, ethanol and H_2 are effective electron donating substrates for the reduction of Pd(II) to Pd(0) by anaerobic granular sludge. Oxidation of formate and H_2 coupled to reduction of Pd(II) to Pd(0) has also been shown in pure cultures of *Shewanella oneidensis*, *Desulfovibrio desulfuricans*, *Desulfovibrio fructosivorans* and *Cupravidus necator*.[9,10,27–29] Biological reduction of Pd(II) with formate or H_2 as electron donors has been proposed to involve electron transfer from the electron donor onto Pd mediated by active [NiFe]-hydrogenases.[9,10,30] Similarly, formate can be converted to H_2 by a membrane-associated enzyme complex, formate hydrogen lyase,[31] and the H_2 formed can promote Pd(II) reduction. This reaction does not imply an energetic problem because H_2 formation by the enzyme formate hydrogen lyase is comparable to ferredoxin-dependent hydrogen formation.[32]

Although involvement of enzymatic conversions on the reduction of Pd(II) by numerous microorganisms is well established, our results indicate that direct chemical reaction of Pd(II) by common microbial metabolites, such as H_2 or formate, can also play an important role in some cases. As shown in Figures 4.1A and 4.1F, rapid removal of Pd(II) was observed in cell-free assays supplemented with H_2 and formate, indicating the ability of both compounds to reduce Pd(II) abiotically. In this regard, it is important to note that previous studies have shown that H_2 at ambient temperature can effectively reduce $PdCl_4^{2-}$ in aqueous solution to Pd(0),[33] and formate and its salts are commonly used in chemical reduction methods to obtain palladium powder.[34] Several studies investigating the effect of microorganisms on Pd(II) reduction by H_2 or formate have reported that reduction did not occur in cell-free controls, suggesting that microbial activity was required for the reaction to occur.[35,36] However, direct chemical reduction of Pd(II) by H_2 and formate has been demonstrated by other authors in cell-free cultures and in assays supplemented with autoclaved cells.[23,27] The results obtained in this study also indicate that, although H_2 or formate can reduce Pd(II) directly, the rate of Pd(II) reduction was higher when the cultures where inoculated (Table 4.2) suggesting that the presence of cells

enhances the reduction of Pd(II) to some extent. Previous studies have demonstrated that cells, including autoclaved cells, can accelerate Pd(II) reduction through a non-enzymatic mechanism by providing nucleation sites and that nucleation is facilitated by Pd(II) interaction with organic functional groups on the cell surface such as amine groups[11,23].

The behavior of ethanol as an electron donor differed from that of H_2 or formate. The results obtained revealed that the reduction of Pd(II) in the presence of ethanol only occurred in the presence of inoculum. The very similar Pd reduction rates determined in inoculated assays spiked with ethanol and with hydrogen (at the concentration expected from the fermentation of the ethanol added) (Table 4.2) suggest that Pd(II) reduction was most likely mediated by H_2 formed from ethanol by acetogens through reaction 4.1.[37] The H_2 formed is an effective reducing agent of Pd(II) as previously discussed.

$$CH_3CH_2OH + H_2O \longrightarrow CH_3COO^- + H^+ + 2H_2 \qquad \Delta G^{o'} = 9.6\ KJ \quad (4.1)$$

4.4.5 Inhibition of acetoclastic and hydrogenotrophic methanogenic activity by palladium

The inhibition of acetoclastic methanogenic activity was tested separately by exposing the anaerobic granular sludge to different concentrations of Pd(II). Acetoclastic methanogens were highly inhibited at very low concentrations of Pd(II) as shown in Figure 4.7A. The 50% inhibiting concentration (IC_{50}) was about 0.96 mg Pd(II) L^{-1}, and nearly complete methanogenic inhibition (92%) was observed at a concentration as low as 3 mg Pd(II) L^{-1}. These results revealed that acetoclastic methanogens are very sensitive towards Pd(II).

Experiments performed to evaluate the inhibitory effect of palladium on hydrogenotrophic methanogenic activity were conducted in two ways: (i) pre-exposing the anaerobic granular sludge to varying concentrations of Pd(II) before H_2 was spiked to minimize palladium reduction and ensure extended exposure to the

ionic palladium species and, (ii) supplementing Pd(II) and H_2 simultaneously. Results are compared in Figure 4.7B and significant differences can be observed. Experiments performed by simultaneous addition of H_2 and Pd(II) did not present inhibition on methanogenic activity even at Pd(II) concentrations as high as 25 or 30 mg Pd(II) L^{-1}. In contrast, experiments where the sludge was pre-exposed to Pd(II) showed significant inhibition as indicated by an IC_{50} value as low as 2.7 mg Pd(II) L^{-1} and near complete methanogenic inhibition (96%) in cultures exposed to 25 mg Pd(II) L^{-1}.

These results obtained in the inhibition bioassays suggests that Pd(II) is the toxic species and that microbial reduction of Pd(II), as readily observed in the H_2 amended cultures, promotes detoxification by formation of poorly bioavailable, non-toxic Pd(0). In the case of the acetoclastic methanogens, the ineffectiveness of acetate as electron donor resulted in extended exposure of the microorganisms to toxic Pd(II), causing irreversible inhibition of the methanogenic activity.

Pd(II) toxicity has also been observed in studies with *Shewanella oneidensis*, a Pd(II)-reducing bacterium.[38] Exposure to a Pd(II) concentration of 10 mg L^{-1} without any electron donor for 1 h was reported to result in a 4 log reduction of culturable cells and a reduction of 60% viable cells based on flow cytometry. In contrast, with the high inhibition observed in the previous studies, Pd(II) was not inhibitory towards the fermentative bacterium *Clostridium pasteurianum* BC1 at initial aqueous Pd(II) concentrations as high as 500 mg L^{-1}.[38,39] Likewise, extended exposure to Pd(II) concentrations up to 25 mg L^{-1} in the bioassays performed in this study with ethanol only caused some mild inhibition of acetogenic microorganisms as indicated by the small decrease in the rate of ethanol consumption with time in cultures that received two spikes of Pd(II) (Figure 4.2). The low inhibition observed it the latter studies is likely due to the rapid detoxification of Pd(II) by formation of Pd(0) in both assays.

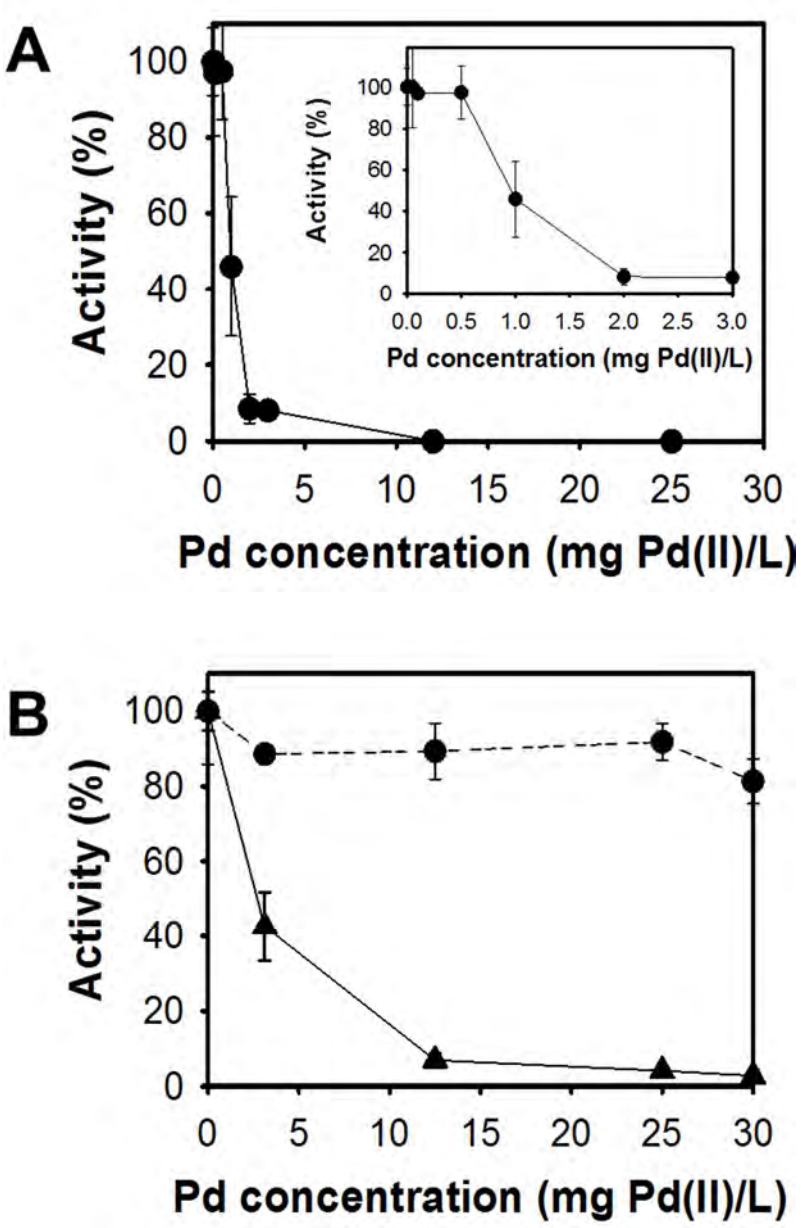

Figure 4.7 Inhibition of the maximum methanogenic specific activity of acetoclastic (A) and hydrogenotrophic (B) microorganisms as a function of the Pd(II) concentration added in assays performed with simultaneous addition of H_2 and Pd(II) (●), and in assays where anaerobic granular sludge was first pre-exposed to Pd(II) for 60 hours and then supplemented with H_2 (▲).

Microbial inhibition by heavy metals has been attributed to disruption of enzyme function and structure by binding of the metals with groups on protein molecules or by replacing naturally occurring metals in enzyme prosthetic groups.[40] Inactivation of the mercapto group in coenzyme M (carrier of methyl groups in methanogenesis) has been proposed to account for the inhibitory impact of many metals on methanogens.[41] Many heavy metals are necessary to drive diverse reactions as they form part of enzymes and they will stimulate microbial activity when present at trace levels. However, palladium is not a natural component of enzymes or cells in anaerobic microorganisms and this investigation demonstrated that Pd(II) has an inhibitory effect on methanogenesis.

4.5 Conclusions

The present study contributes to a better understanding of the ability of anaerobic granular sludge for metal reduction and the information found provides new insights into the inhibitory effects of Pd(II) on methanogenic microorganisms. The ability of methanogenic granular sludge to reduce Pd (II) to elemental palladium was demonstrated. These findings indicate the potential of anaerobic granular sludge bioreactor technology for the reduction and recovery of palladium from aqueous waste streams.

4.6 References

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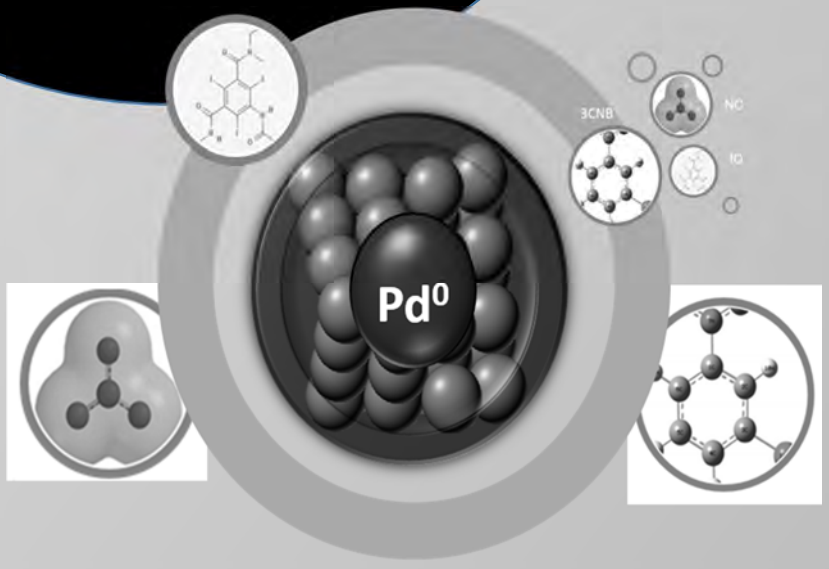
CHAPTER 5

Continuous removal and recovery of palladium in an upflow anaerobic granular sludge bed (UASB) reactor



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5.1 Abstract

The aim of this study was to investigate the feasibility of utilizing a continuous-flow anaerobic granular sludge bed (UASB) reactor for the removal of palladium (II) from aqueous streams and the recovery of this precious metal as biogenic Pd(0). Anaerobic methanogenic granular sludge has been recently shown to reduce Pd(II) in the presence of a suitable electron donor leading to the formation of biomass-associated Pd(0).

Two laboratory-scale UASB reactors were fed with ethanol or acetate, in order to compare their performance in terms of Pd removal and reduction. Pd(II) was added in two periods of increasing concentration, 5 mg Pd(II) L^{-1} and then 15 mg Pd(II) L^{-1}. Extensive removal of Pd, (up to 99%), was attained in both reactors. X-ray diffraction (XRD) and energy-dispersive X-ray spectroscopy (EDS) analysis confirmed significant differences in the content of Pd(0) in the biomass, depending on the electron donor used. The main mechanisms of Pd(II) removal when acetate and ethanol were used as electron donor were biosorption and reduction to Pd(0) by biogenic H_2 released as fermentation product, respectively it is possible to apply a continuous process for Pd reduction and recovery using a methanogenic consortium.

5.2 Introduction

Platinum group metals have attractive properties such as resistance to chemical corrosion over a wide temperature range, high melting point, high mechanical strength and good ductility, reasons for what they are extensively used in industrial processes. Among them, demand and use of palladium (Pd) is important because this metal is used for different purposes, such as automobile catalysts, electronics, industrial catalysts, circuitry, dental alloys, jewelry and lately in the treatment of cancer.[1–3] Palladium is a very scarce element that accounts for only 0.0000015% of the earth's crust. In recent years demand and production of Pd has increased rapidly,

with the concomitant increase in market price.[4–6] Due to its increasing importance and projected supply risks in the short-term, palladium has been identified among several elements that are critical to one or more new energy-related technologies (e.g., catalysts in fuel cells that have many potential applications, including hydrogen fuel and hybrid cars).[7] Under this scenario the recovery of this metal from Pd containing-streams has turned into an important issue since this would translate into a solution for its increasing demand, it would help to decrease capital cost and reduce environmental impact.[8]

Different methods to recover precious metals from aqueous solutions are used by industries: solvent extraction, ion exchange, evaporation, cementation and chemical precipitation.[9] Many of these methods have some drawbacks such as incomplete metal removal, high reagent and/or energy requirements, and limited applicability when treating diluted effluents.[10] Biological techniques have emerged to offer a new and efficient alternative for metal recovery and many reports have focused on Pd(II) reduction to Pd(0) by using pure cultures with concomitant production of valuable Pd(0) nanoparticles.[11–15] More recently the use of mixed cultures has also been explored. Two very recent studies reported reduction of palladium(II) with formation of biogenic Pd(0) by a sulfate-reducing enrichment culture[16] and by a fermentative consortium.[17] Most of these studies have been performed in batch mode under anaerobic conditions, and until now, palladium recovery using a continuous reactor has not been reported.

Practical application of palladium recovery at the industrial scale would require a continuous flow method and a cost-effective technology. Since the cost attributed to the culturing and handling of the bacteria can amount to as much as €1000 kg^{-1} cell dry weight,[18] the use of mixed microbial consortia is an attractive option since the metal recovery process can operate under non aseptic conditions which facilitate process control and reduce maintenance and operation costs.[19,20] Upflow anaerobic sludge blanket reactors (UASB) and related granular sludge reactor technologies, which are the most commonly applied high-rate anaerobic wastewater treatment system,[21–25] offer interesting perspectives for application in palladium recovery since granular biomass has characteristics that makes it ideal as a support material for the

biogenic Pd(0). Microbial granules have a stronger aggregate structure and better settleability compared to dispersed biomass,[26] thus, washout of Pd(0) particles in the reactor effluent would be minimized since the Pd formed by microbial reduction is largely associated to the biomass.[17] Furthermore, the use of granular sludge reactors would facilitate the recovery of the biomass associated Pd for future reuse.[17] The broad experience available for the full-scale application of anaerobic granular sludge bioreactor technologies would also facilitate process scale-up of a new process for metal removal. UASB reactors operated under sulfate-reducing conditions have been previously applied to remove and recover heavy metals,[27,28] however, there are not reports on the application of the process for metal removal under non-sulfate reducing conditions.

The aim of this study was to evaluate the feasibility of laboratory-scale UASB reactors inoculated with methanogenic granular sludge to reduce Pd(II) to biogenic Pd(0) that can be recovered for further reuse. Two bioreactors were operated in parallel to compare the effectiveness of two different electron donating substrates, acetate or ethanol. The inhibitory effect of palladium on the activity of acetoclastic and hydrogenotrophic methanogens was also investigated.

5.3 Materials and methods

5.3.1 Source of anaerobic granular sludge

The methanogenic anaerobic granular sludge was obtained from a full scale UASB reactor treating brewery wastewater (Mahou, Guadalajara, Spain). The content of volatile suspended solids (VSS) was 4.77% of the wet weight. The anaerobic sludge was stored at 4°C under a N_2 atmosphere before use.

5.3.2 Continuous-flow reactors

Two laboratory-scale UASB reactors (Internal diameter= 4.1 cm, volume= 270 mL) were operated in parallel. Each reactor was inoculated with 10 g VSS L^{-1} of anaerobic granular sludge. The reactors were continuously fed with a pH-7.2 mineral medium containing (g L^{-1}): $NaHCO_3$ (2.5), K_2HPO_4 (0.25), $MgCl_2 \cdot 6H_2O$ (0.2), NH_4Cl (0.1), $CaCl_2 \cdot 2\ H_2O$ (0.01), yeast extract (0.01), and 0.25 mL L^{-1} of a trace elements

solution that contained (mg L^{-1}) H_3BO_3 (50), $FeCl_2 \cdot 4H_2O$ (2000), $ZnCl_2$ (50), $MnCl_2 \cdot 4H_2O$ (50), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (50), $AlCl_3 \cdot 6H_2O$ (90), $CoCl_2 \cdot 6H_2O$ (2000), $NiCl_2 \cdot 6H_2O$ (50), $CuCl_2 \cdot 2H_2O$ (30), $NaSeO_3 \cdot 5H_2O$ (100), EDTA (1000), and HCl (1 mL L^{-1}). Resazurin was added (1 mL L^{-1}) from a stock solution of 200 mg L^{-1} as a redox indicator of anaerobic conditions. Sulfate was excluded from the medium to avoid precipitation of Pd as PdS. The influent of UASB reactor 1 (R1) was supplemented with ethanol (0.11 g chemical oxygen demand (COD) L^{-1}) and the influent of UASB reactor 2 (R2) with acetate (0.07 g COD L^{-1}). The addition of acetate was based on the amount expected from the fermentation of the ethanol present in the influent of R1.

The reactors were run at room temperature (23±2°C) and fed continuously using a peristaltic pump. Both reactors were covered with aluminum foil to prevent the growth of phototrophic microorganisms.

The reactors were operated in three periods. In period 1, which spanned the initial 19 days of operation, the influent of R1 and R2 was supplemented with acetate and ethanol, respectively, but without addition of Pd(II). In Period 2, day 20 to 40, the influent of both reactors was also supplemented with 5 mg Pd(II) L^{-1} (added as Na_2PdCl_4 in HCl 0.01%). In Period 3 (day 41-54), the concentration of Pd(II) in the influent of both reactors was increased to 15 mg L^{-1}. During these periods, the empty bed hydraulic retention time (HRT) of both reactors was maintained constant at 7.8 ± 0.1 h.

The performance of the reactors was monitored by measuring the pH value, the concentrations of acetate, ethanol and soluble Pd in influent and effluent. The gas outlet of each reactor was connected to a Tedlar bag to collect the produced biogas. Additionally, the specific methanogenic activity (SMA) of the anaerobic sludge in the reactors was determined at the end of period 1 and at the end of the experiments to evaluate the potential inhibitory effect of Pd(II) on the methanogenic process.

5.3.3 Batch acetoclastic and hydrogenotrophic methanogenic activity assays

The acetoclastic and hydrogenotrophic methanogenic activity of the sludge was evaluated in shaken batch assays. The assays were carried out at the end of period 1 (after 20 days of feeding with only acetate and ethanol) and at the end of the exposure to palladium in both reactors (day 54). All assays were carried out in glass serum bottles (160 mL) containing 25 mL of the same medium used in the reactors. The bottles were inoculated with the anaerobic granular sludge directly obtained from the reactors to a final concentration of 1.5 g VSS L^{-1}. The medium and the headspace were flushed with a gas mixture containing N_2/CO_2 (80:20, v/v) to ensure anaerobic conditions. The substrate was added as acetate (1 g COD L^{-1} as CH_3COONa) or H_2 (0.5 atm, supplied as H_2/CO_2 (80:20, v/v)). The bottles were incubated in the dark at 30±2°C in an orbital shaker at 110 rpm. Headspace samples (100 µL) were obtained periodically and analyzed for CH_4 content until the maximum theoretical methane production was reached.

The maximum SMA, (g CH_4-COD g^{-1} VSS d^{-1}) was calculated from the slope of the CH_4 content versus time graph, as the mean value of duplicate assays corresponding to the samples at the end of period 1. The activity determined at the end of the experiment (day 53) was normalized to the activity of the non-inhibited control as follows:

$$\text{Normalized methanogenic activity (\%)} = (SMA_{\text{end of experiment}}/SMA_{\text{control}}) \times 100$$

5.3.4 X-ray diffraction analysis

X-ray diffraction (XRD) analysis of the microbial samples collected from both reactors at the end of the experiment were carried out in an Philips X'Pert MPD diffractometer at a wavelength of 1.5406 A Cu-Kα1; generator settings of 40 kV and 40 mA; scan step size of 0.02 from 20° to 100° 2θ. Samples were prepared as described previously by Lloyd et al. (1998).[29]

5.3.5 Scanning electron microscopy

Sludge samples obtained from both reactors were prepared for scanning electron microscopy (SEM) by fixation in 2.5% glutaraldehyde for 30 min, washed with 2 changes in 0.1 M PIPES buffer (pH 7.4) and further fixed in 2% osmium tetroxide for

15 min. Pellets were washed three times in deionized water and dehydrated through a graded ethanol series (50, 70, 90 and 100%) immersed in hexamethyldisilazane (HMDS) for 5 min, and air dried. Samples were coated with platinum-gold. The images were collected on Hitachi S-4800 Type II / Thermo NORAN NSS EDS. SEM images were collected at 30 kV.

5.3.6 Analytical techniques

The concentration of soluble Pd in the influent and effluent of both reactors was analyzed by inductively coupled plasma-optical emission spectroscopy (ICP-OES Optima 2100 DV, Perkin Elmer, Waltham, MA, USA) at a wavelength of 340.5 nm. Samples were analyzed after filtering successively through a 0.45-μm and 0.025-μm membrane filter (VSWP, Millipore, Billerica, MA, USA). The detection limit for Pd was 10 μg L^{-1}.

The concentrations of ethanol and acetate were measured by gas chromatography (7890A GC system, Agilent Technologies, Santa Clara, CA, USA) using a fused silica Stabilwax®-DA column (30 m length, 0.53 mm ID, Restek Corporation, State College, PA, USA) and a flame ionization detector. The temperature of the injector port and the detector were set at 280°C. The initial temperature of the column was 100°C and it was increased up to150°C at a rate of 8°C per min. Helium was used as the carrier gas (5.2 mL min^{-1}), and air and hydrogen as flame source. The injection volume was 1 μL.

The methane content in headspace samples was analyzed by gas chromatography using a Hewlett–Packard 5890 Series II system (Agilent Technologies) fitted with a Restek Stabilwax-DA fused silica capillary column (30 m length, 0.53 mm ID) and a flame ionization detector. The temperature of the column was 140°C and the injector and detector temperatures were 180 and 250°C, respectively. Helium was used as the carrier gas at a flow rate of 18 mL min^{-1} with a split flow of 85 mL min^{-1}.

5.4 Results and Discussion

5.4.1 Removal of Pd(II) in continuous-flow UASB reactors

The COD removal attained by the two reactors as a function of time is shown in Figure 5.1. During the initial period of operation (days 1 to 19) only ethanol (R1) or acetate (R2) was added to each reactor in order to adapt the sludge to the substrate and to evaluate the performance of the bioreactors in the absence of palladium addition. The COD removal efficiencies achieved by both reactors during this period was very high, averaging 99±1.2%.

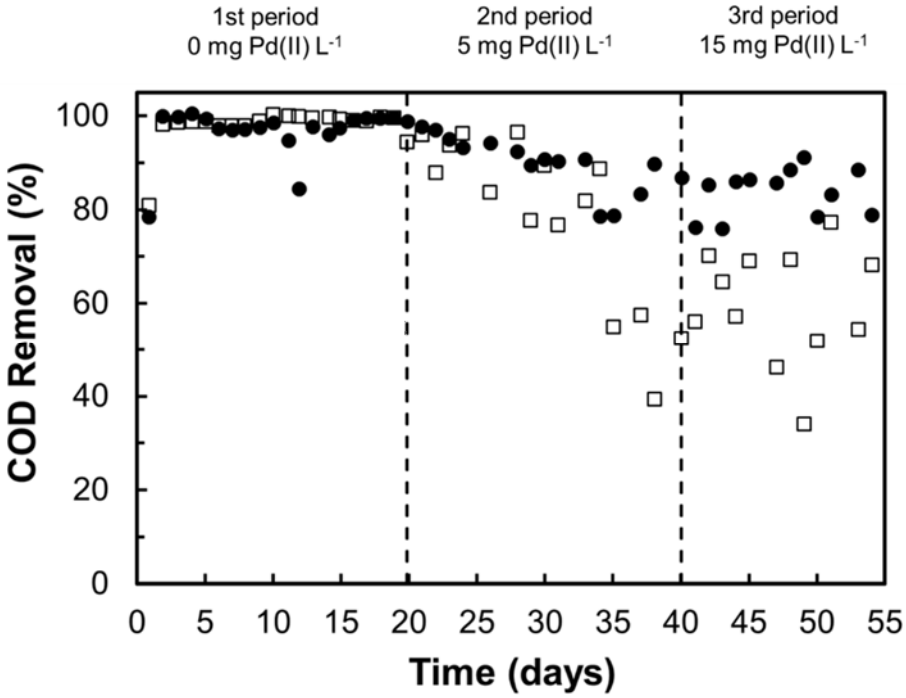

Figure 5.1 Percentage of COD removal in the UASB reactors R1 fed with ethanol (●) and UASB reactor R2 fed with acetate (□).The dashed vertical lines on days 20 and 40 indicate the beginning of Pd(II) addition in the reactors at concentrations of 5 and 15 mg L□□, respectively.

Between days 20 to 40 (period 2), the influent of both reactors was supplemented with Pd(II) (5 mg L^{-1}). As shown in Figure 5.1, the COD removal efficiency of both reactors was affected by the presence of Pd(II). The performance of the reactor fed acetate (R2) was particularly impacted and the COD removal efficiency attained by the reactor dropped after 10 days of Pd(II) addition from 99.0% to 59.9±12.2%. The decrease in COD removal was due to the accumulation of acetate in the system (up to 20 mg acetate-COD L^{-1}), suggesting inhibition of acetoclastic methanogens by palladium. In contrast with these results, the performance of the reactor fed with ethanol (R1) was less affected by the presence of Pd(II) and the average COD

removal efficiency only decreased from 99.0% to 86.7±5.1%. The observed decrease in the treatment performance of reactor R1 started after 14 days of Pd(II) addition and was due to incomplete removal of acetate, indicating inhibition of acetoclastic methanogens. No ethanol was detected in the effluent of R1 during this period. In spite of the observed decrease in the COD removal efficiency, both reactors provided high levels of Pd(II) removal, averaging 93.9 ± 0.8% for R1 and 90.7 ± 2.5% for R2 (Figure 5.2) at the end of the second operation period (days 35-40).

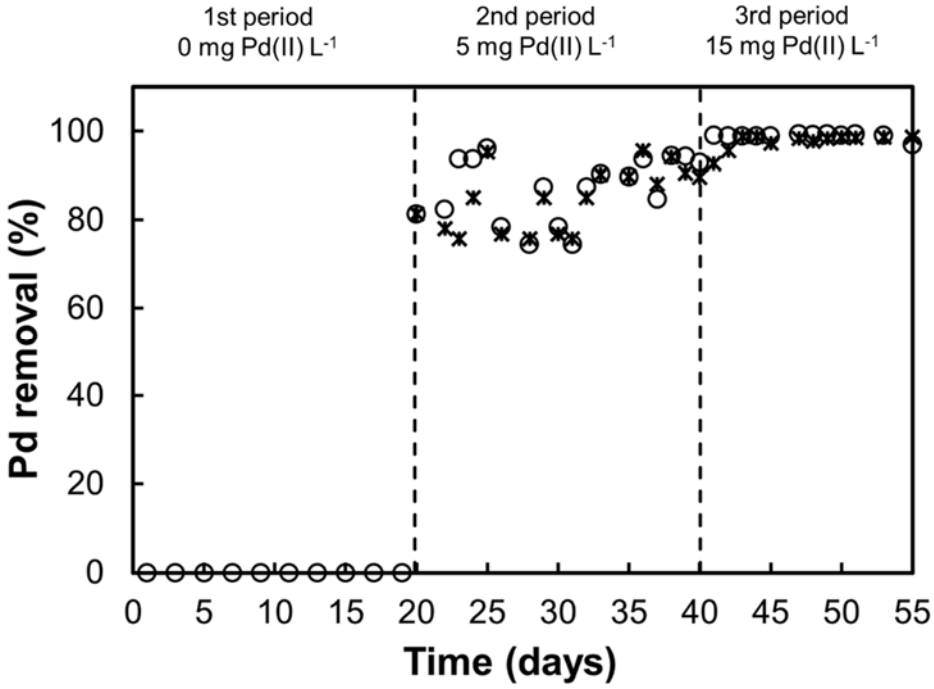

Figure 5.2 Percentage of Pd removed as a function of time in the UASB reactor R1 fed with ethanol (○) and UASB reactor R2 fed with acetate (✕) as a function of the time of operation. The dashed vertical line indicates the time when the Pd(II) concentration added to the influent of the reactors was modified.

During the final period of operation (days 40 to 55) the concentration of Pd(II) in the influent of both reactors was increased to 15 mg L^{-1}. During this period, the average COD removal efficiency of R2 remained low, averaging 57.4 ± 15.1%. In contrast, the average COD removal in the reactor fed ethanol (R1) averaged 83.9 ± 5.0%. The

Pd(II) removal efficiency in both reactors was very high, averaging 98.9 ± 0.7% for R1 and 97.7 ± 1.8% for reactor R2 (Figure 5.2).

The obtained results revealed significant differences in the performance of the reactors depending on the electron donor used, which could be mainly related to different mechanisms of Pd removal. In R1, fed with ethanol, the mechanism of Pd(II) removal is via reduction stimulated by biogenic H_2 originated from ethanol fermentation. This concept can convert the process in a self-sustained strategy for Pd(II) reduction and recovery as valuable Pd(0) since it has been demonstrated that H_2 is an excellent reducing agent and electron donor for the microbially mediated reduction of Pd.[12,14,30,31]

In the case of R2, although acetate is a good substrate for methanogenesis, the collected evidence indicates that it was a very poor electron donor for Pd reduction. Nonetheless, R2 provided extensive removal of Pd(II), which could be mainly due to a biosorption mechanism since it has been demonstrated that Pd(II) has a high tendency to sorb onto microbial biomass.[32–34] Thus, the results obtained demonstrated that Pd(II) removal can be promoted by different mechanisms depending on the electron donor supplied: Pd(II) reduction, conditioned to the use of a suitable reducing agent or sorption to biomass when a poor electron donor is used.

Microbial biomass samples obtained from both reactors at the end of the operation were analyzed by SEM and XRD. SEM images of both samples revealed that microorganisms were covered by aggregates, but EDS analysis indicated that Pd was present in more abundance in the product obtained from R1 (Figure 5.3).

These results were further confirmed by XRD analysis validating that the product was Pd(0) in both samples (Figure 5.4), with the Pd metal being relatively more abundant in the sample corresponding to R1 (fed with ethanol) (Figure 5.3A).

XRD patterns were indexed to ICDD card 046-1043 (fcc palladium syn) and it was found to correspond to the characteristic diffraction peaks of crystalline Pd(0). These results confirm that in R1 Pd was effectively reduced to Pd(0) whereas in R2 adsorption of Pd(II) was an important removal mechanism. Nonetheless XRD results indicate the presence of some Pd(0) in the biomass of R2, suggest that some Pd

reduction may have linked to the degradation of organic compounds released from biomass decay. Endogenous metabolism was likely promoted as consequence of starvation conditions once acetate consumption stopped as a result of Pd(II) inhibition.[35]

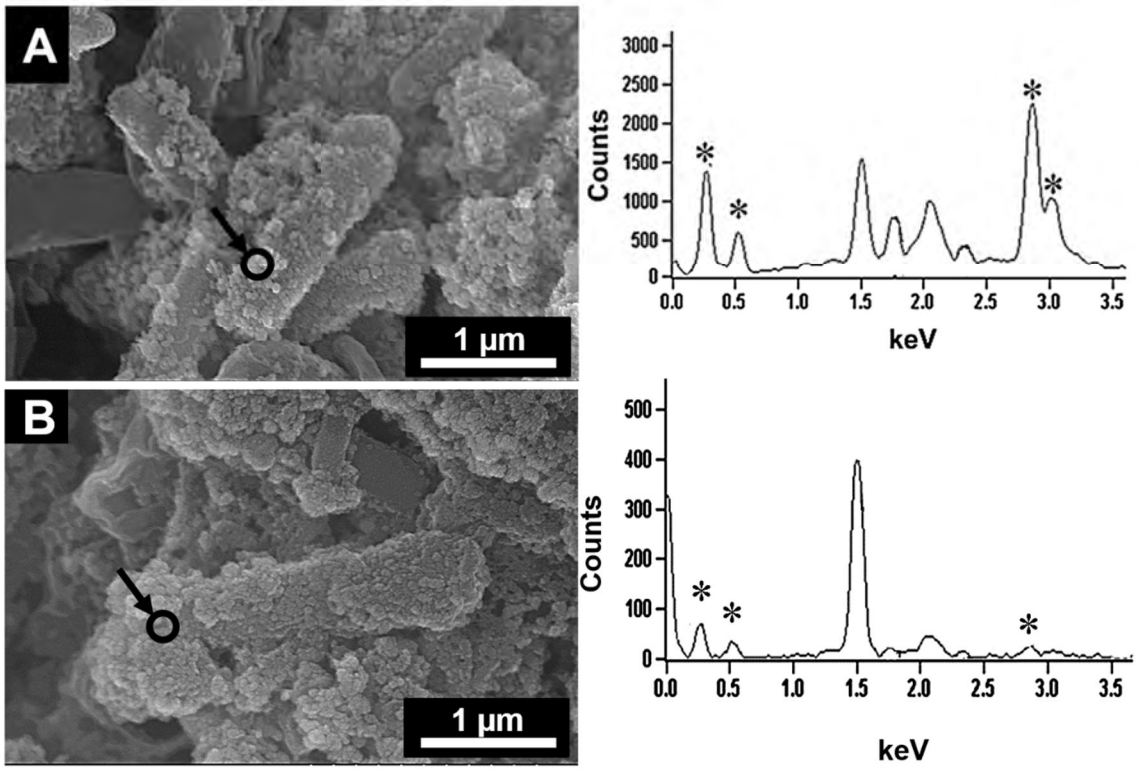

Figure 5.3 On the left, SEM images of microbial samples obtained from the UASB reactor R1 fed with ethanol (A) and UASB reactor R2 fed with acetate (B) after 55 days of operation. On the right, energy-dispersive X-ray spectroscopy diagrams corresponding to the samples shown in the SEM images. The circle and arrow indicate the point of analysis. The asterisks (*) indicated the peaks corresponding to palladium as detected in the EDS analysis.

Furthermore, some fermentative bacteria (e.g., *Citrobacter braaki*), which are an important population in anaerobic granular sludge, have been reported to be able to reduce Pd by producing H_2 during fermentation processes and, subsequently, H_2 chemically reduce Pd(II) to Pd(0).[31,36]

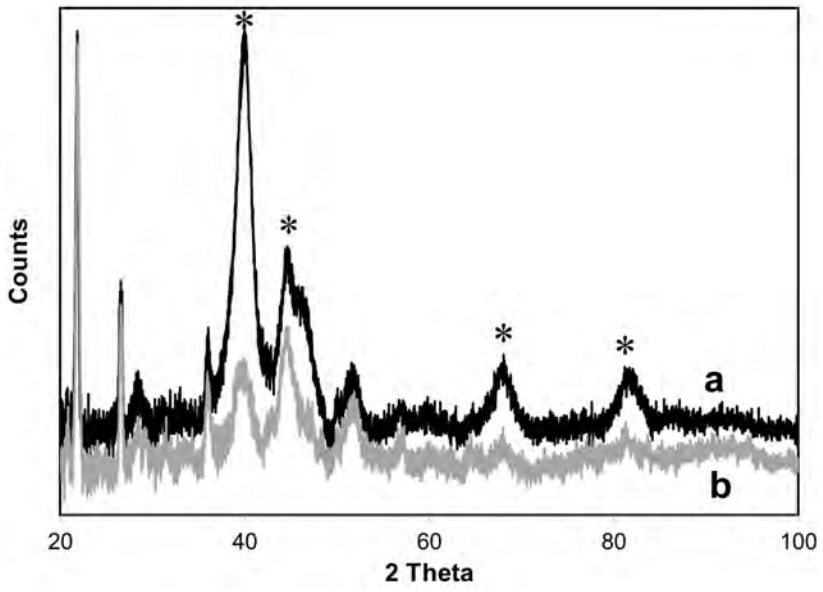

Figure 5.4 XRD patterns corresponding to the microbial samples obtained from the UASB reactor R1 fed with ethanol (a) and UASB reactor R2 fed with acetate (b) after 35 days of operation with an influent containing Pd(II). The asterisks (*) showed the diffraction peaks for Pd metal at the expected positions according to crystallographic data (ICDD card 046-1043).

5.4.2 Inhibitory effect of Pd(II) on methanogenesis

The maximum SMA of the anaerobic granular sludge in both bioreactors was determined at the end of the adaption time (period 1) and after extended exposure to Pd(II) (day 55) to assess the potential inhibitory impact of Pd(II) on acetoclastic and hydrogenotrophic methanogens. The results obtained in these activity tests are shown in Figure 5.5.

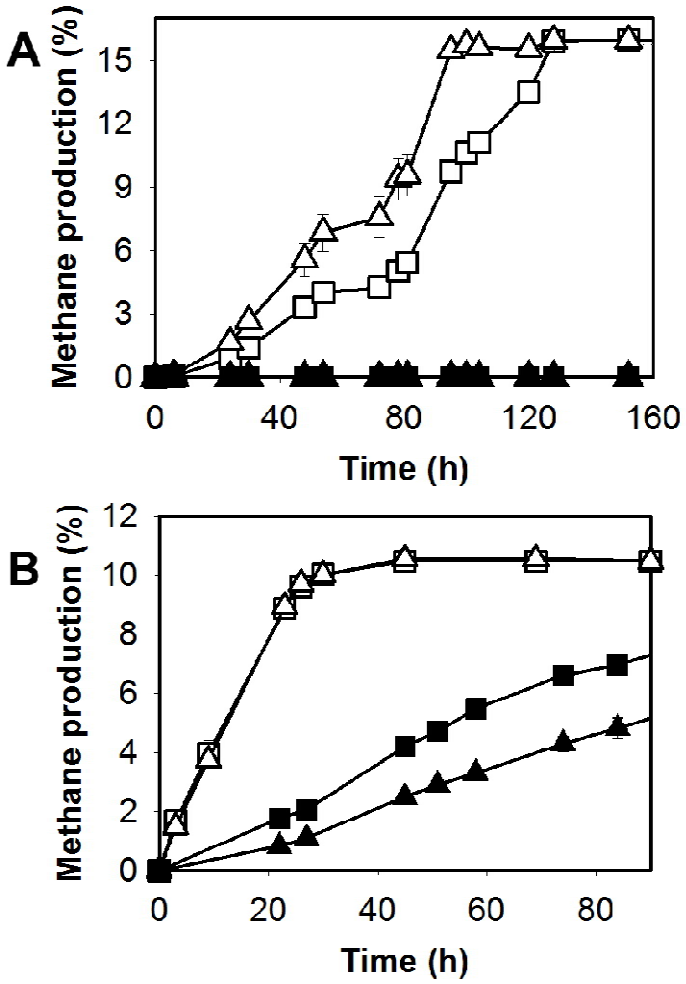

Figure 5.5 Methanogenic acetoclastic activity (A) and hydrogenotrophic activity (B) of anaerobic granular sludge samples obtained before Pd(II) addition to the UASB reactor R1 fed with ethanol (□) and UASB reactor R2 fed with acetate (∆), and after 35 days of operation of the reactors R1 (■) and R2 (▲)with Pd(II). (Where error bars are not shown, the error was smaller than the symbols).

Results from these incubations showed similar values for hydrogenotrophic SMA in R1 and R2 before Pd addition, 1.01 and 1.02 g CH_4-COD g^{-1} VSS d^{-1}, respectively (Table 5.1).

Table 5.1. Specific acetoclastic and hydrogenotrophic methanogenic activity (mgCH_4-COD mgVSS^{-1} d^{-1}) values for UASB reactor R1 fed with ethanol and UASB reactor R2 fed with acetate calculated from samples taken before and after Pd addition.

	Specific methanogenic activity (mg CH_4-COD mg^{-1} VSS d^{-1})			
	Hydrogenotrophic		Acetoclastic	
	Before Pd addition	After Pd addition	Before Pd addition	After Pd addition
R1	1.013 ± 0.24	0.250 ± 0.02	0.811 ± 0.08	0.010 ± 0.02
R2	1.019 ± 0.17	0.169 ± 0.02	1.040 ± 0.08	0.010 ± 0.03

The acetoclastic activity of the granular sludge in R1 (fed with ethanol) was slightly lower compared to R2 (fed with acetate), with values of and 0.81 and 1.04 g CH_4-COD g^{-1} VSS d^{-1}, respectively. At the end of the reactors operation period (day 55), the methanogenic activity of the granular sludge in both reactors was determined again for comparison. The results obtained showed that the acetoclastic methanogenic activity was almost completely lost in both reactors (Table 5.1). A lower but marked decrease in the hydrogenotrophic activity of the biomass was also observed as indicate by activity values for R1 and R2 that were 69.5% and 80.4% lower, respectively, compared to those determined in the biomass prior to the addition of Pd(II).

These results demonstrated that acetate-utilizing methanogens are more sensitive than H_2-utilizing methanogens. The latter microorganisms have the capacity to avoid inhibition effects through Pd(II) reduction by H_2 produced during fermentation processes. Once Pd(II) is reduced to Pd(0), it seems to be much less toxic than Pd(II). Pd(II) toxicity has also been observed in studies with *Shewanella oneidensis*, a Pd(II)-reducing bacterium.[37] The main reason of this difference is attributed to the ionic form of Pd in the medium, Pd(II) is the soluble form and Pd(0) is insoluble, and as it is known only metals in soluble, free form are toxic to the microorganisms thus Pd(0) is not expected to cause a toxic effect.[38–40] This could explain the better COD removal efficiency observed in the reactor fed with ethanol.

These results indicate that long-term exposure to high Pd(II) concentrations could lead to complete loss of the methanogenic activity.

5.5 Implications

The present work constitutes the first report documenting continuous palladium recovery from synthetic wastewater using UASB reactors. Our results confirmed the high capacity of this process to remove Pd(II) from synthetic wastewater. Reduction of Pd(II) and retention of Pd(0) formed by association with the anaerobic granular sludge was the main mechanism of removal of palladium. This strategy could be feasible for recovering palladium and other precious metals from industrial wastewaters, such as metal-processing and acid rock drainage. The Pd(0) obtained under these conditions could be easily recovered and reused in different fields. Simple and feasible recovery techniques can be applied when the removal of the biomass from the already produced Pd(0) is required, such as solvent extraction or burning.[41]

One of the main applications would be the use of Pd(0)-enriched biomass as bio-catalyst for the biodegradation of recalcitrant pollutants as it has previously been shown that biogenic Pd(0) (bio-palladium) has great catalytic capacity to promote the biodegradation of contaminants, such as polychlorinated biphenyls, trichloroethylene, diatrizoate, among others.[12,31,42]

The present work represents the first study demonstrating the feasibility for achieving palladium recovery from contaminated water using methanogenic granular sludge in a continuous bioreactor process. The results obtained confirmed that extensive removal of Pd (up to 99%) was attained in UASB reactors utilizing ethanol or acetate as substrate.

The main mechanisms participating in palladium recovery were demonstrated to be dependent on the electron donor used: effective removal was promoted by formation of biomass-associated biogenic Pd(0) using ethanol as electron donor and, in the case of the acetate-fed bioreactor, likely also by Pd(II) biosorption.

These findings indicate that anaerobic granular sludge bed reactor technology is an option for recovery of Pd from waste streams and provide knowledge that can help to design operational strategies to achieve Pd reduction and recovery.

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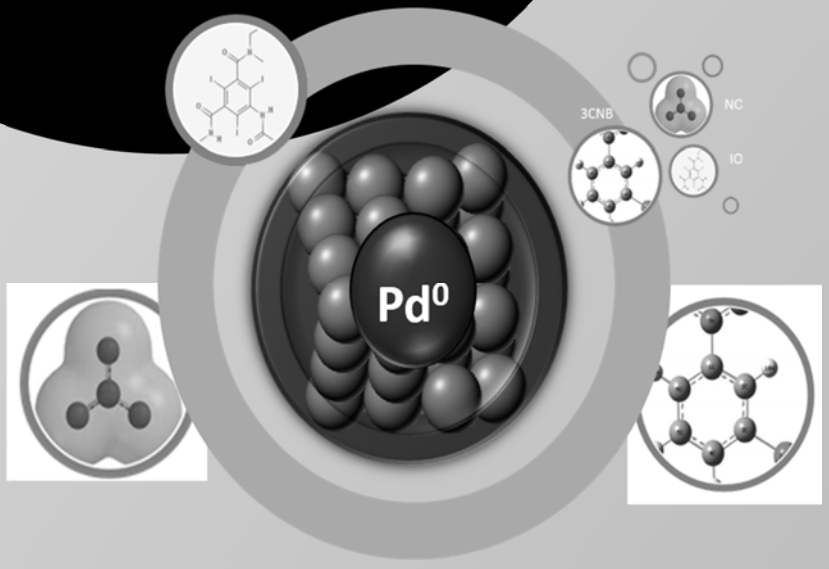
CHAPTER 6

Immobilization of biogenic Pd(0) in anaerobic granular sludge for the biotransformation of recalcitrant halogenated pollutants in UASB reactors



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6.1 Abstract

The capacity of anaerobic granular sludge to reduce Pd(II), using ethanol as electron donor, in an up-flow anaerobic sludge bed (UASB) reactor was demonstrated. Results confirmed complete reduction of Pd(II) and immobilization as Pd(0) in the granular sludge. The Pd-enriched sludge was further evaluated regarding biotransformation of two recalcitrant halogenated pollutants: 3-chloro-nitrobenzene (3CNB) and iopromide (IOP) in batch and continuous operation in UASB reactors. The superior removal capacity of the Pd-enriched biomass when compared with the control (not-exposed to Pd) was demonstrated in both cases. Results revealed 80% of IOP removal efficiency after 100 h of incubation in batch experiments performed with Pd-enriched biomass whereas only 28% of removal efficiency was achieved in incubations with biomass lacking Pd. The UASB reactor operated with the Pd-enriched biomass achieved 81±9.5% removal efficiency of IOP and only 61±8.3% occurred in the control reactor lacking Pd. Regarding 3-CNB, it was demonstrated that biogenic Pd(0) promoted both nitro-reduction and dehalogenation resulting in the complete conversion of 3-CNB to aniline while in the control experiment only nitro-reduction was documented. The complete biotransformation pathway of both contaminants was proposed by HPLC-MS analysis evidencing a higher degree of nitroreduction and dehalgenation of both contaminants in the experiments with Pd-enriched anaerobic sludge as compared with the control. A biotechnological process is proposed to recover Pd(II) from industrial streams and to immobilize it in anaerobic granular sludge. The Pd-enriched biomass is also proposed as a biocatalyst to achieve the biotransformation of recalcitrant compounds in UASB reactors.

6.2 Introduction

There is an increasing concern about halogenated aromatic compounds because of their toxicity, fate and persistence in the environment [1,2] which are mainly related to their chemical characteristics. These compounds are produced in large scale since they are intensively used as pharmaceuticals, herbicides, fungicides, insecticides, flame retardants, intermediates in organic synthesis, and for many other

applications.[3] Among them 3-chloronitrobenzene (3-CNB) and iopromide (IOP) are compounds of particular interest as their biodegradation is challenging because of their high persistency in the environment and due to the formation of transformation products, which are more toxic than their precursors, reasons that encourage the development of new treatment techniques.

Reduction of water contaminants by palladium-based catalysts has emerged as a promising alternative as this catalyst can activate H_2 to promote, for instance, reductive transformations of a number of compounds such as oxyanions, halogenated alkenes and aromatics.[4] However, as palladium is extensively used with different purposes in industrial processes, its demand and production has increased as well as its market price.[5] For that reason, many efforts have been focused on studying alternatives to reclaim this metal from wastes and to regenerate Pd(0) by reduction of soluble Pd(II).[6]

Waste materials containing high metal concentrations can serve as secondary raw materials [7], for example spent catalysts, electronic scrap leachates, acidic leachates, etc., are potential sources of Pd recovery.

In this context, biological strategies have been explored and positioned as feasible options to conventional methods with this purpose. Regarding this concept, several studies have reported on the use of pure microbial cultures for achieving Pd(II) reduction for the recovery of Pd(0).[8–13]

Microbially driven reduction of Pd(II) is now considered as a feasible option since it implies a process with less aggressive conditions, fairly easy manipulation and avoid the production of toxic byproducts.[14] Moreover, the use of mixed microbial consortia can offer advantages over pure cultures including easy process control and low operational costs since operation in aseptic conditions is not necessary and consortia have the ability to adapt to minor changes in conditions and promote synergistic interactions.[15–17] Furthermore, if a methanogenic consortia is provided with an adequate electron donor it would promote Pd(II) reduction to Pd(0) via biogenic hydrogen produced from fermentation and this H_2 can be used to activate the catalyst once Pd(0) has already been produced, in this way the process would be self-sustained.[18] For that reason the aim of this work was to develop a new option

for the recovery of palladium from industrial streams based on the capacity of methanogenic granular sludge to reduce Pd(II) and to immobilize biogenic Pd(0) in the granular biomass. The Pd(0)-enriched granular sludge was tested as a bio-catalyst to achieve the biodegradation of the halogenated recalcitrant pollutants 3CNB and IOP in batch and in continuous upflow anaerobic sludge blanket (UASB) reactors. Biodegradation pathway for both contaminants is proposed based on HPLC-MS analysis.

6.3 Material and methods

6.3.1 Source of anaerobic granular sludge

Anaerobic granular sludge was obtained from a full-scale UASB reactor treating brewery wastewater (Sonora, Mexico). The content of volatile suspended solids (VSS) was 6.15% of the wet weight. The anaerobic sludge was stored at 4°C before use.

6.3.2 Enrichment of anaerobic granular sludge with Pd(0) in UASB reactors

Two laboratory-scale UASB reactors (volume 160 mL) were inoculated with 10 g VSS L^{-1} of anaerobic granular sludge and operated in parallel. The mineral medium used to feed the reactors was composed of (g L^{-1}): $NaHCO_3$ (2.5), K_2HPO_4 (0.25), $MgCl_2{\cdot}6H_2O$ (0.2), NH_4Cl (0.1), $CaCl_2{\cdot}2\ H_2O$ (0.01), yeast extract (0.01), and 0.25 mL L^{-1} of a trace elements solution that contained (mg L^{-1}) H_3BO_3 (50), $FeCl_2{\cdot}4H_2O$ (2000), $ZnCl_2$ (50), $MnCl_2{\cdot}4H_2O$ (50), $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (50), $AlCl_3{\cdot}6H_2O$ (90), $CoCl_2{\cdot}6H_2O$ (2000), $NiCl_2{\cdot}6H_2O$ (50), $CuCl_2{\cdot}2H_2O$ (30), $NaSeO_3{\cdot}5H_2O$ (100), EDTA (1000), and HCl (1 mL L^{-1}). Anaerobic indicator resazurin was added to the medium and sulfate was excluded from the medium composition to avoid interference with Pd as it can be precipitated as PdS (if sulfate reduction takes place). Reactors were operated at room temperature of 21±5°C and protected from light to prevent growth of phototrophic microorganisms or photodegradation of the tested contaminants.

During the first 30 days of operation, the influent of both reactors was supplied with 0.14 g chemical oxygen demand (COD) L^{-1} of ethanol to achieve a constant COD removal of 100%. After this period and during 20 days the influent of one reactor (R-

Pd) was supplemented with 3 mg Pd(II) L^{-1} (added as Na_2PdCl_4 in HCl 0.01%) while the other reactor remained fed with ethanol but without addition of Pd(II). The former contained the enriched-anaerobic sludge with Pd(0) (R-Pd) and the later, the reactor not exposed to Pd(II), was considered as the control reactor (R-ctrl). The hydraulic retention time (HRT) of both reactors was maintained constant at 8 h. The performance of both reactors was monitored by measuring the pH value and COD removal. The produced biogas of each reactor was collected and analyzed as described below.

6.3.3 Biotransformation of iopromide and 3-chloronitrobenzene in UASB reactors

Reactors R-Pd and R-ctrl were operated in parallel as described above. Influent of the reactors was supplemented with 3-CNB or IOP depending on the period of operation. In a first operation cycle, 3-CNB degradation was tested and performed in three periods. In period 1 (days 1-20), 0.1 mM of 3-CNB and 0.14 g ethanol-COD L^{-1} were supplemented to the influent of both reactors. During period 2, the concentration of 3-CNB was increased to 0.3 mM and the electron donor was supplied at 0.24 g ethanol-COD L^{-1} (days 21-34). In period 3, the 3-CNB concentration remained at 0.3 mM, but no external donor as ethanol was added (days 35-37). Samples were analyzed at selected time intervals during the reactors operation to determine the concentration of 3-CNB, COD in the influent and effluent and intermediary products formed as a result of 3-CNB degradation. After this period, sludge samples were taken from both reactors to perform 3-CNB batch biodegradation experiments as described below.

After this cycle, with the purpose of allowing the recovery of the microbial activity, both reactors were fed with ethanol as the only carbon source until COD removal remained constant at 100% (10 days) and a second operation cycle was immediately started.

In the second operation cycle, the influent of the reactors was supplemented with IOP to give a concentration of 0.2 mg iopromide L^{-1} (22 days).

Samples were taken from both bioreactors at selected time intervals to analyze IOP and its transformations products as well as to determine COD removal. After this period, sludge samples were taken from both reactors to perform IOP batch biodegradation experiments as described immediately.

6.3.4 Batch biotransformation experiments

Biodegradation of 3-CNB and IOP was evaluated in batch experiments. Batch assays were carried out, separately, at the end of cycle 1 (after 37 days of feeding with 3-CNB) and at the end of the cycle 2 (after 22 days of feeding with iopromide) of both UASB reactors. All assays were carried out in duplicate for each experimental treatment in 120-mL glass serum bottles capped with inert stoppers with 100 mL of total liquid volume of the same medium used in UASB reactors.

Bottles were inoculated with anaerobic granular sludge obtained from both reactors to yield a concentration of 2 g VSS L^{-1}. Subsequently, liquid and headspace were flushed with N_2/CO_2 (80:20, v/v) to ensure anaerobic conditions. An appropriate volume of ethanol was added to give a final concentration of 0.14 g chemical oxygen demand (COD) L^{-1}.

Depending on the batch biodegradation assay, incubations were supplied with 3-CNB (0.1 and 0.3 mM) or IOP (150 µg L^{-1}). Bottles were covered with aluminum foil and incubated in the dark to prevent photodegradation of the tested contaminants.

Samples were analyzed at specific time intervals to monitor the change in the compounds concentrations.

6.3.5 Analytical techniques

6.3.5.1 Analysis of 3-CNB, IOP and their transformation products

To document microbial reduction of Pd(II), samples were analyzed by inductively coupled plasma-optic emission spectroscopy (ICP-OES, Varian 730-ES) as indicated by Pat-Espadas et al.[19]

The concentration of 3-CNB was measured spectrophotometrically at 265 nm using a Thermo Scientific UV–vis (Genesis 10uv model). The concentration of 3-chloroaniline (3CA) and aniline were measured by high-performance liquid chromatography (HPLC). Phosphate buffer containing (g L^{-1}): $NaH_2PO_4 \cdot H_2O$ (10.86), $Na_2HPO_4 \cdot 2H_2O$ (5.38) and ascorbic acid (0.2) freshly added was used during the determination to prevent oxidation. Samples from reactors and batch experiments were analyzed after filtering through a 0.22 μm membrane filter. The HPLC (AgilentTechnologies, 1200 series) was equipped with a column synergi 4U Hydro-RP 80R (250·4.60 mm, 4 micron) from Phenomenex. Two microliter of sample was injected with an autosampler. The carrier liquid, composed of 60% of deionized water and 40% acetonitrile, was pumped at a flow rate of 1 mL min^{-1}. Compounds were detected at 40°C at a wavelength of 280 nm with a diode array detector.

IOP concentration was measured by HPLC using the equipment described previously and detected at 25°C at a wavelength of 280 nm with a diode array detector

Identification of transformation products of both contaminants was performed in a Varian® 500-MS ion trap mass spectrometer, electrospray ionization with voltage of 90V and mass-to-charge (m/z) range of 100 to 1500 m/z. COD was determined according to standard methods.[20]

6.4 Results

6.4.1 Enrichment of anaerobic granular sludge with Pd(0)

During the start-up period both reactors were supplemented only with ethanol in order to adapt the sludge to the substrate (30 days) achieving 98 ± 3% of COD removal efficiency in both reactors. After this period, the influent of one reactor (R-Pd) was supplemented with Pd(II) (3 mg Pd(II) L^{-1}) for 20 days. This UASB reactor (R-Pd) achieved 99±0.8% of Pd removal during this operational period. COD removal efficiency was also monitored and no negative impact was observed in the reactor supplemented with Pd since COD removal remained up to 98% as well as in the control UASB reactor not exposed to Pd. XRD analysis of sludge from R-Pd confirmed that Pd(II) was effectively reduced to Pd(0) in R-Pd (data not shown).

Characteristic diffraction peaks of crystalline Pd(0) particles at 2θ values of 40°, 47°, and 68° were identified according to ICDD card 05-0681 (fcc palladium syn).

6.4.2 Biotransformation of 3-CNB in UASB reactors

The reduction of 3-CNB by anaerobic sludge (R-ctrl) and anaerobic sludge enriched with Pd(0) (R-Pd) was evaluated in continuous mode using the previously stabilized UASB reactors. COD and 3-CNB removal and reduction efficiencies attained by the bioreactors as a function of time are shown in Figure 6.1. Both UASB reactors were initially tested for their capacity to degrade 0.1 mM of 3-CNB and the reduction efficiency achieved by both reactors in that period was 96.4±2.4% (Figure 6.1A). After this period, the concentration of 3-CNB was increased to 0.3 mM and the average reduction accomplished was similar to the previous period averaging 94.4±3%.

These results showed no differences with respect to 3-CNB reduction in both reactors, which were operated with a HRT of 8 h, regardless the presence of P(0) in R-Pd. However, COD removal efficiency of the control reactor was particularly affected (Figure 6.1B) when an increment of 3-CNB concentration was applied. In period 1 the control reactor (R-ctrl) achieved an average COD removal of 93.9±8 % (with a minimum value of 80.5%). In contrast, the performance of the Pd(0)-enriched reactor (R-Pd) was less affected, averaging 99.3 ±2 % of COD removal. During period 2, COD removal efficiencies in R-ctrl underwent important fluctuations with an average removal of 79.5±24.1%, whereas in R-Pd it was 85.9±21.3%. At the end of this period the COD removal was as low as 57% in R-ctrl and 81% in R-Pd. In the next period of operation (period 3) no ethanol was supplemented as external electron donor and both reactors were fed only with 0.3 mM of 3-CNB. Results revealed that 3-CNB reduction efficiency drop to ~70% in both reactors. These results suggest that addition of ethanol as co-substrate is essential to achieve high reduction efficiencies of 3-CNB.

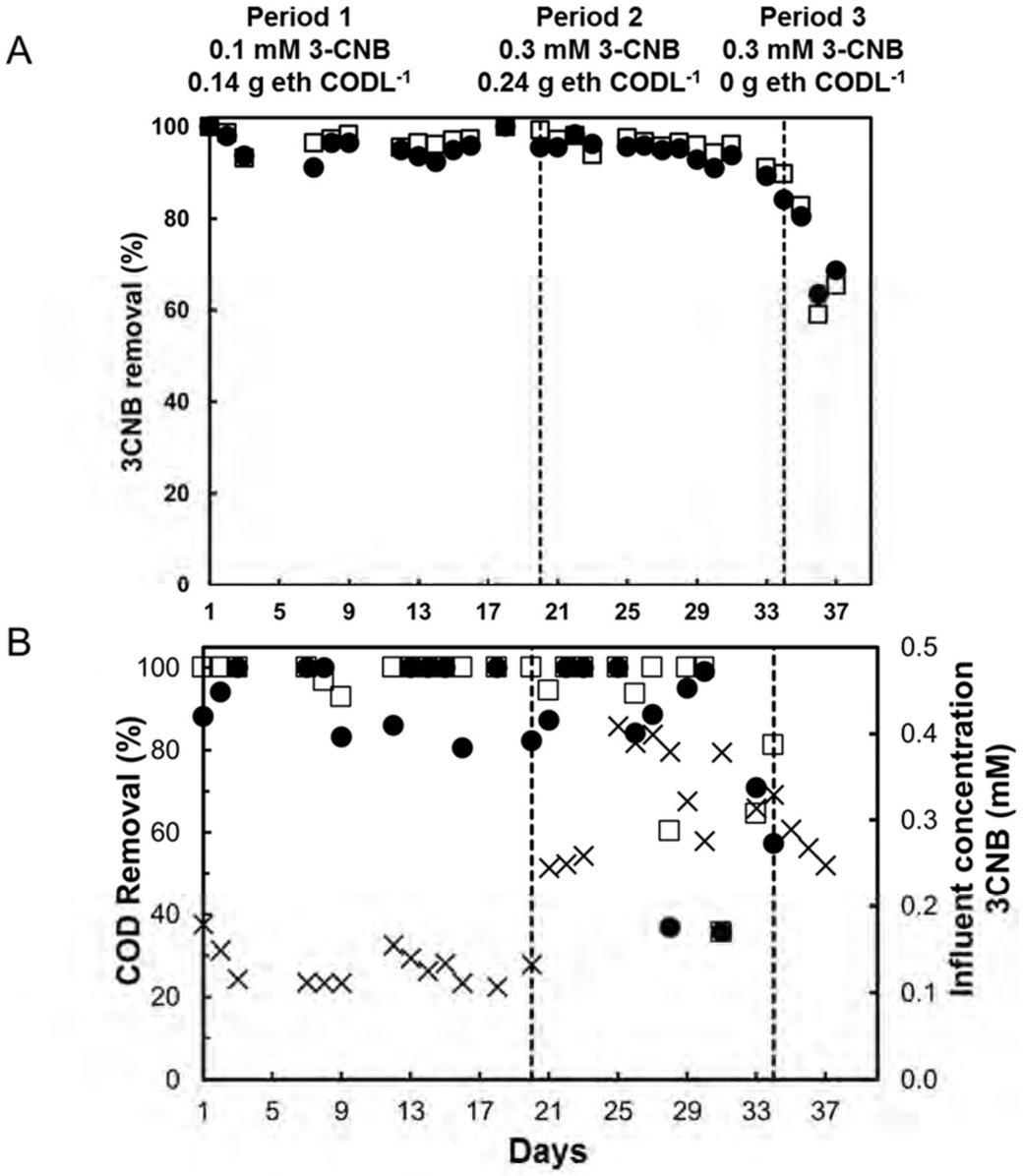

Figure 6.1 Percentage of 3-CNB removal in the UASB reactors with Pd(0) enriched-anaerobic sludge, R-Pd (□), and anaerobic sludge not exposed to Pd, R-ctrl (●) (A) and COD removal achieved in R-Pd (□) and R-ctrl (●) at different 3-CNB influent concentrations (X) as function of the time operation(B). The dashed vertical lines in days 21 and 34 indicate the beginning of the different operation periods as indicated.

6.4.3 Batch experiments of 3-chloronitrobenzene biotransformation

In order to elucidate the biotransformation pathway of 3-CNB followed by both reactors, batch incubations were performed at the end of the operational period. Batch experiments were carried out to test differences between the biomass not exposed to Pd and the enriched-anaerobic sludge with Pd(0). A concentration of 0.3 mM of 3-CNB was tested (Figure 6.2) and 83.5±0.5% of 3-CNB reduction was achieved in both experimental treatments after 10 h of incubation. The results confirmed that the presence of Pd(0) in R-Pd did not promote a faster 3-CNB removal, the maximum degradation rate was 5 mg 3-CNB L^{-1} h^{-1} (0.032 mM 3-CNB h^{-1}).

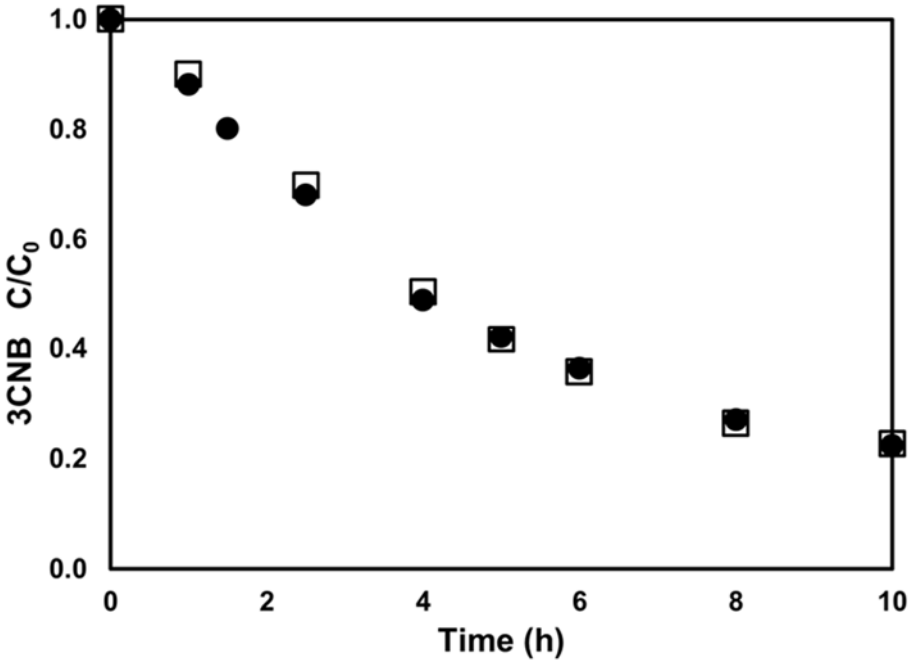

Figure 6.2 Time course of the conversion of 3-CNB at concentrations of 0.3 mM in batch incubations performed with Pd-enriched anaerobic sludge (●) and sludge not exposed to Pd (□).

Nevertheless, further analysis performed by HPLC and HPLC-MS revealed significant differences regarding the extent of transformation of 3-CNB between biomass samples derived from R-ctrl and R-Pd.

Samples analyzed by HPLC after the incubation experiments (results not shown) showed that aniline was the main product obtained in incubations performed with Pd-enriched biomass with a recovery of 97.2±1.4% (this result suggest that, at this time of sampling, only about of 2.8% was 3-CA) which implies a higher level of transformation involving reduction of the nitro group and dehalogenation. Nevertheless the occurrence of compounds such as nitroso– and hydroxylamine– is not dismissed since these derivatives could be formed as intermediaries.[21]

In contrast, in control incubations lacking Pd, 3-CA was the first product detected with a recovering percentage of 2.2±0.3%, which suggests that the rate of intermediates occurrence varies between both experimental conditions. Furthermore, a higher degree of degradation was dependent on the presence of Pd(0). From these results it was supposed that 3-CA is the first transformation product that could be detected and in a further step this compound was transformed into CA. However the final products of the incubation were analyzed by HPLC-MS to identify the final transformation products and suggest the degradation pathway.

Results obtained from HPLC-MS analysis at the end of the experiments revealed two ion peaks at *m/z* 96.8 and *m/z* 78.8 in both samples (peaks were present in both samples but the percentages of abundance were higher for both compounds in R-Pd samples). The experimentally obtained mass (m/z 96.8, negative ionization) suggests a similar structure to aniline; the structure proposed was an unsaturated aromatic ring (containing 1 double bond) with the amine substituent, 1-amine-2-cyclohexene. This product could be formed by hydrogenation of the double bonds of the benzene ring catalyzed by Pd.[22,23] The structure proposed for the other identified compound (*m/z* 78.8) was a benzene ring which could be formed by loss of the amine substituent.

According to the results obtained by HPLC and HPLC-MS it was proposed a complete pathway of 3-CNB biotransformation, which is suggested to end in the formation of two compounds (Figure 6.3). In a first step 3-CNB would be transformed via nitro-reduction to the corresponding aromatic amine (3-CA). In a second step, 3-CA is converted to aniline by reductive dehalogenation and, according to batch

results, in the presence of Pd catalyst this reaction is faster and more favored than in incubations with the sludge not exposed to Pd.

In a last step the transformation goes further and aniline can be converted into two main products, benzene or 1-amine-2-cyclohexene. The formation of these products implies deamination of the molecule or the saturation of the benzene ring by hydrogenation, reactions that could be mainly promoted by Pd-catalyst.

The pathway in control experiments is suggested to be the same, but occurring at slower rates and obtaining low yield as compared to incubations with anaerobic sludge enriched with Pd.

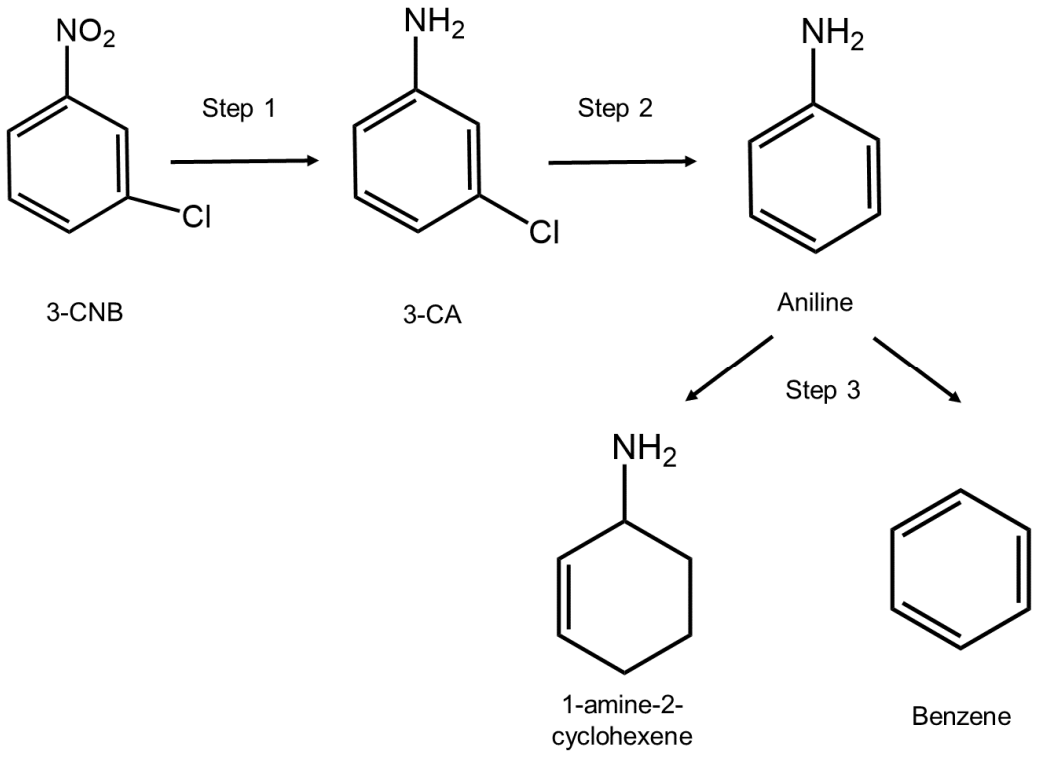

Figure 6.3 Proposed pathway for the biotransformation of 3-CNB. Step 1, nitro-reduction; step 2, reductive dehalogenation; step 3, deamination of the molecule or saturation of the benzene ring by hydrogenation.

6.4.4 Biotransformation of iopromide in UASB reactors and batch experiments

The biotransformation of IOP was also evaluated in both reactors (R-Pd and R-ctrl) at constant concentration of 200 μg L^{-1} during 22 days, both reactors were supplied with ethanol. The performance of both reactors regarding iopromide removal is shown in Figure 6.4A.

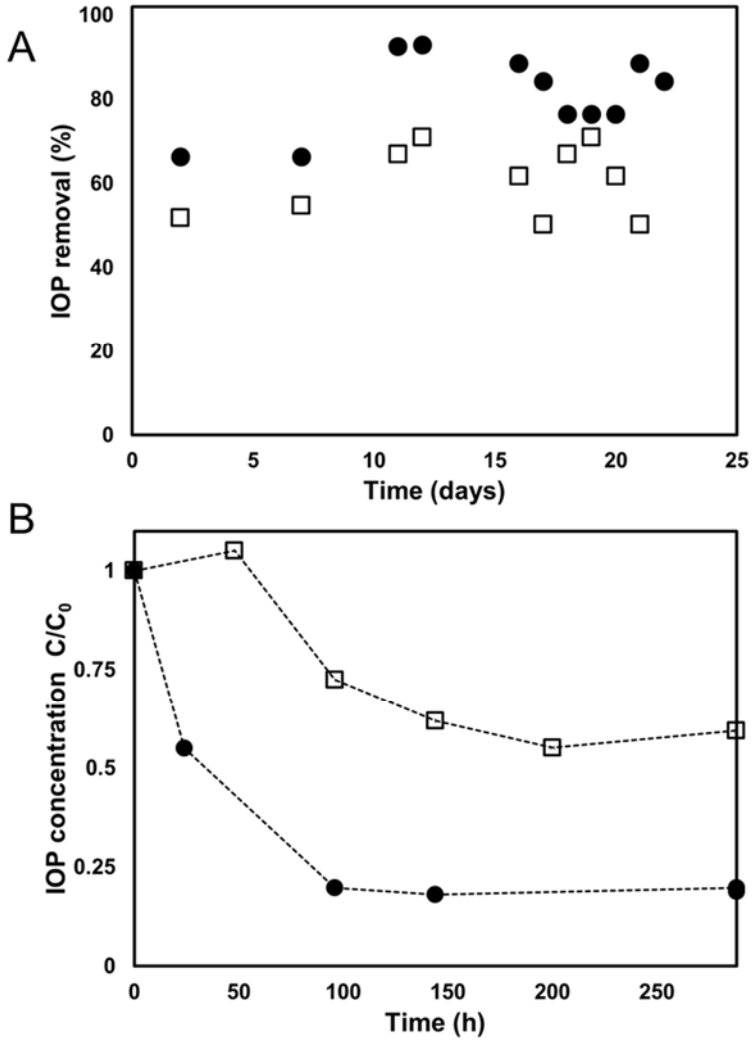

Figure 6.4 Percentage of iopromide removal efficiency achieved in the UASB reactors with anaerobic sludge not exposed to Pd, R-ctrl (□) and with Pd(0) enriched-anaerobic sludge, R-Pd (●) as function of time operation (A) and time course of the conversion of iopromide in batch incubations performed with Pd-enriched biomass (●) and biomass not exposed to Pd (□) (B).

The UASB reactor containing anaerobic sludge enriched with Pd (R-Pd) achieved an average removal of 81.1±9.5% whereas only 60.6 ±3.3% was achieved in R-ctrl. From these results it can be concluded that R-Pd was more efficient regarding IOP removal than the control reactor at a HRT of 8 h, which could be attributed to the catalytic input of immobilized biogenic Pd(0). Furthermore, no significant variations in the average COD removal efficiency were detected; R-Pd achieved 90.5 ± 12.6% while R-ctrl attained 85.9 ± 4.7%. With the aim of determining in more detail the fate of IOP, batch experiments were run and the time course of the IOP concentration is shown in Figure 4B. It was necessary 100 h to remove 80% of the IOP in Pd-enriched anaerobic sludge incubations. In contrast, only 28% of IOP removal was achieved in the control experiment during the same experimental period and the maximum removal efficiency was 50% after 288 h of incubation. The maximum rate calculated for both experiments was 1.12 and 0.51 μg IOP $L^{-1}h^{-1}$, for Pd-enriched anaerobic sludge and control sludge incubations, respectively, which indicate that immobilized Pd(0) has an important catalytic effect during the biotransformation of IOP.

6.4.5 Biotransformation pathway of iopromide

Samples from both incubations (batch), Pd-enriched anaerobic sludge and control not exposed to Pd, were further analyzed by HPLC-MS in order to elucidate the biotransformation pathway. Six transformation products (TPs) were detected under these experimental conditions and their possible participation in the biotransformation pathway was explained according to identified structures as it is shown in Figure 6.5, based on available literature and molecular weight of the different structures. Each TP is briefly described in the following lines supported in previous reports in literature.

Structure of TP 633.8 (elemental composition $C_{17}H_{22}O_7N_3I_2$) was proposed according to literature, which indicates that this intermediate could be obtained after the loss of H_2O of the reported TP 651.0 (elemental composition $C_{17}H_{23}O_8N_3I_2$).[24] This TP 651.0 is reported to be produced from the loss of CHI which indicate the loss of one iodine atom and the N-demethylation in side chain A.[24] Moreover, the

structure of the TP identified as TP 715.9 was proposed according to Perez et al.[25] This structure indicates the cleavage of the amide bond in side chain B with the elimination of water. TP 707.8 is proposed to be formed by the loss of the amide side chain (A or B) which could also occur after other transformation reactions (oxidation and decarboxylation) of the parent compound. The structure of this TP is exemplified in Figure 6.5 as the loss in side chain A, and this TP has a similar structure to TP704 reported by Eversloh et al. [26] Furthermore, the cleavage of the C-N bond at side chain A of TP 715.9 results in the formation of TP 627.8. This fragmentation pathway is similar to that proposed by Schulz et al.[27], for the fragmentation pathway of TP 731 B to give m/z 627. The loss of the amide bond at side chain B of TP 633.8 and rearrangement of side chain A result in TP 551 as presented in Figure 6.5. This TP structure can also result from the decarboxylation and N-demethylation at side chain A of TP 715.9 as showed in Figure 6.5. These structures are similar to those proposed by Pérez et al. and Gros et al.[24,25] Finally, the structure of TP 387.0 (elemental composition $C_{13}H_{12}IN_2O_4^+$) is suggested to result as a consequence of loss of part of side chain A in the amide bond and by the elimination of HI, which results in the formation of a five membered ring structure. Similar fragmentation pathways are suggested in the literature.[27]

Figure 6.5 shows the complete pathway proposed for IOP biotransformation in incubations with Pd-enriched anaerobic sludge. Results obtained from the incubations performed with anaerobic sludge (control) revealed that the main TPs present were 633.8 and 715.9. From these findings it can be concluded that the presence of Pd-catalyst promote a higher degree of dehalogenation which is evidenced by the formation of TPs with low m/z such as TP 551.0 and TP 387.0.

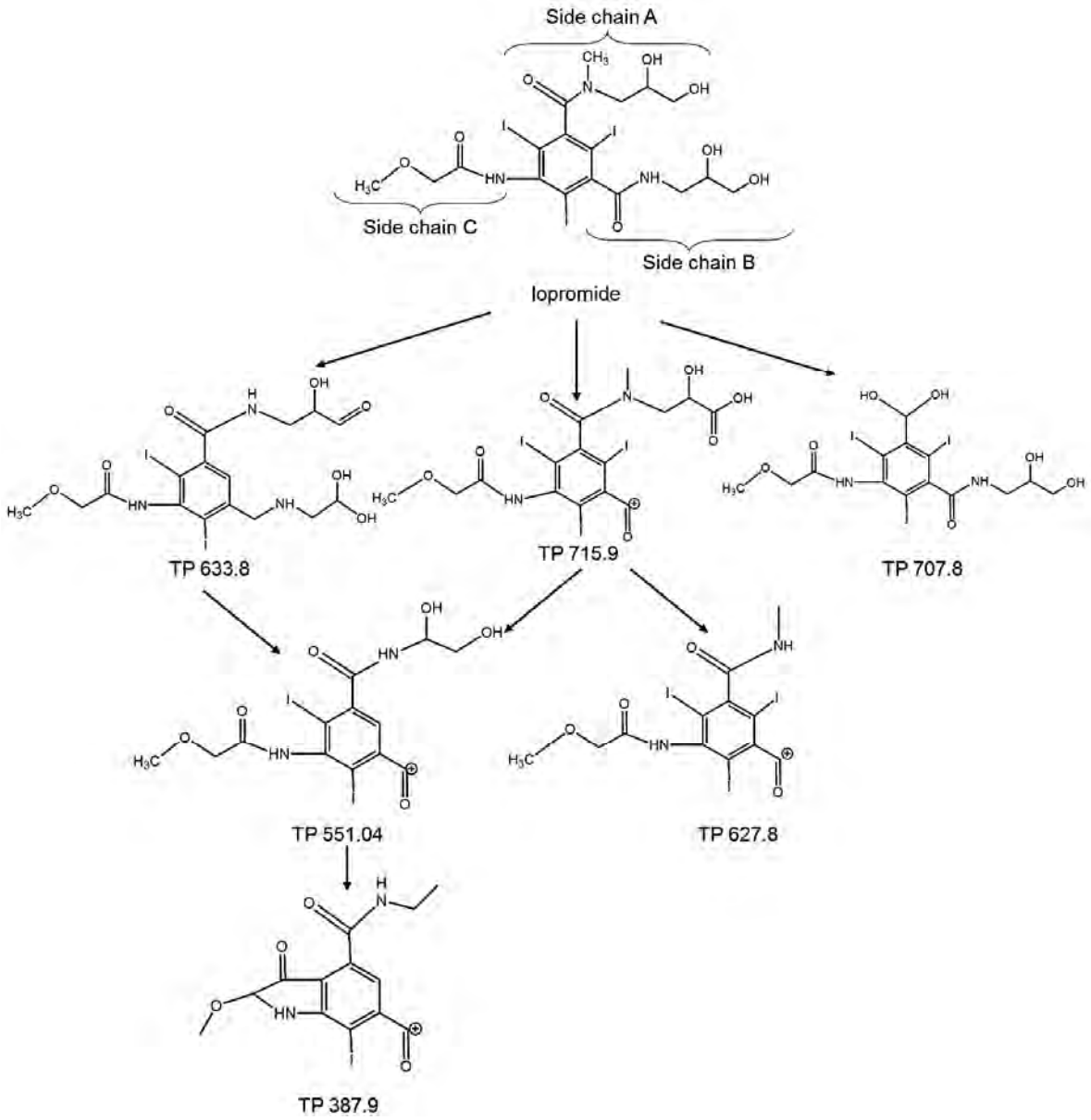

Figure 6.5 Proposed biotransformation pathway of iopromide and final products.

6.5 Discussion

The present work reports for the first time the recovery of Pd(II) from synthetic wastewater and its immobilization as biogenic Pd(0) in anaerobic granular sludge for its subsequent application as catalyst in the biotransformation of recalcitrant contaminants in a UASB reactor. Biogenic Pd(0) has previously been demonstrated as an effective catalysts to promote the biotransformation of a variety of recalcitrant contaminants.[28–32] However, an immobilizing mechanism to retain the nano-catalyst

in bioreactors is demanded in order to preserve the catalytic performance. Different strategies have been explored to prevent leaching of the bio-catalyst from bioreactors, such as membrane systems (hollow and plate membranes)[33–35] and encapsulation in polymeric matrices (polyurethane, polyacrylamide, alginate, silica, etc)[31] involving important costs.[8] Moreover, Suja and colleagues[36] demonstrated that interaction between biogenic Pd(0) and aerobic granular sludge promoted the reduction of *p*-nitrophenol and Cr(VI). Our previous study demonstrated that it is possible to reduce Pd(II) to Pd(0) in UASB reactors using anaerobic granular sludge, which promoted its immobilization.[18]

To the best of our knowledge the present work is the first demonstration that it is possible to use immobilized biogenic Pd(0) in anaerobic granular sludge for its application to enhance the biotransformation of recalcitrant halogenated compounds in anaerobic bioreactors. Experimental results showed high removal efficiency of the two model contaminants considered (IOP or 3-CNB) and the catalytic input of immobilized Pd(0) promoted a greater extent of biotransformation of both contaminants.

The compounds explored in this study belong to the group of aromatic halogenated compounds and their chemical structure is constituted by a benzene ring with halogen substituents, which are responsible of their differences. In general, these chemicals are candidates of reductive transformations, for instance reductive dehalogenation occurs most readily under strict anaerobic conditions (≤-400 mV) and requires a reducing auxiliary substrate.[37] Both compounds, IOP and 3-CNB, are considered persistent with accumulation of toxic metabolites.

Biotransformation of 3-CNB is challenging because of the strong electron attracting of the nitro- and chloric-substituents.[38–40] There are a few studies concerning 3-CNB biotransformation by methanogenic consortia. Colunga et al., 2015[41] reported high inhibitory effects and poor reduction of 3CNB at concentration of 0.5 mM under methanogenic conditions, suggesting to apply an adequate loading rate of this type of contaminant in UASB reactors to avoid inhibition of the microbial consortia. Other studies concerning *p*-CNB have been reported in anaerobic conditions using zero valent iron (dosage of 1 $g \cdot L^{-1}$, for *p*-CNB of 0.28 mM)[42] and a novel bioelectrode-

UASB coupled system (99% removal of *p*-CNB with transformation rate of 0.328 h^{-1} for concentration 0.19 mM).[43] Moreover 3-CNB has great resistance to biodegradation (more than p-CNB) because of the meta-position of the substituents in the molecule[44], besides the nature of chloro and nitro substituents, themselves.

On the other hand, IOP, a tri-iodinated compound used as X-ray contrast medium, has been detected in domestic and hospital wastewaters [45–47], surface waters[47], groundwater and bank infiltrates.[27] This compound would pass conventional wastewater treatment plant since sorption onto sludge or sediments is not expected because it is very polar, highly water-soluble (0.97 mol L^{-1}, 770 g L^{-1}) and it has low lipophilicity (log K_{OW} = -2.33).[2,48] Some treatment systems, such as advanced oxidation[49], aerobic[50,51], stable nitrifying enrichment cultures[52], fungus[24], electrochemical[26] and reductive treatment with catalysts[53] have been examined to determine their potential application. Among these strategies, just the electrochemical treatment of reverse osmosis concentrates reported up to 96% of mineralization after about 7.5 h.[26] Metal-catalyzed treatment with H_2-5% Pd/Al_2O_3 system achieved complete hydrodeiodination of the compound with the formation of a final stable organic product.[53] In this study, two model halogenated recalcitrant pollutants were biotransformed into simpler structures which would constitute the first step towards their mineralization, since complete oxidation of the produced metabolites, in the case of 3-CNB, is expected by aerobic processes in a second stage [21]. Regarding IOP, since is the triiodinated aromatic ring structure which imparts high resistance to oxidation and high recalcitrance under anaerobic conditions it is expected that a simpler structure (mono-halogenated) could be could be more susceptible to mineralization [54].

In this context, the biotechnological process proposed allows not only the recovery of Pd(II) from industrial wastewaters, but also its immobilization as bio-catalyst in anaerobic granular sludge. Furthermore, immobilized Pd(0) was demonstrated as an effective catalyst promoting high removal capacity and greater extent of biotransformation of the model recalcitrant pollutants evaluated. The main advantage of this treatment concept is that no supporting material is needed to immobilize biogenic Pd(0) as it can be retained in the granular sludge matrix. This

strategy can be self-sustained by using an adequate electron donor (ethanol) that could lead to the release of biogenic-H_2, as fermentation product, which serve as reducing agent for Pd(II) reduction[32] and as an activating agent for the immobilized catalyst.

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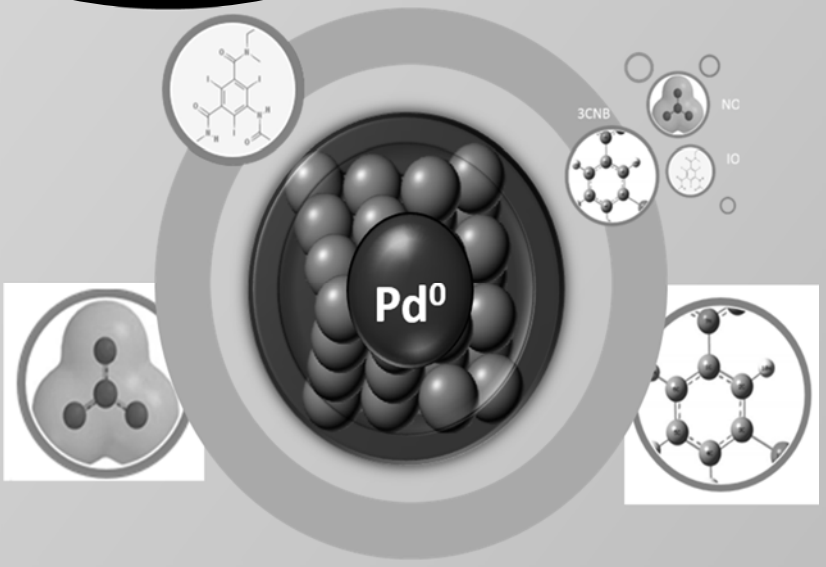
CHAPTER 7

General Discussion



7.1 Introduction

Biological methods have emerged as a feasible option for metal recovery and metallic nanoparticles production.[1,2] The importance of biological methods is the easy manipulation and low energy requirements besides to be considered as a non-toxic procedure.[2] Many efforts have been done in this field regarding control of size, morphology shape and monodispersity of nanoparticles by manipulating parameters, for instance, biomass:metal ratio and composition of the reaction medium. Moreover, investigations have focused in the use of different types of microorganisms to achieve a practical process to be applied with favorable results. Hence, this dissertation was mainly focused on exploring the capacity of a pure strain and methanogenic consortia for their capacity to reduce Pd(II) and recover it as Pd(0) nano-catalyst.

On the one hand, *G. sulfurreducens* strain PCA is a mesophilic bacterium that can grow by acetate oxidation coupled to reduction of different electron acceptors[3] and in this study it was demonstrated that *G. sulfurreducens* can couple the oxidation of acetate to the reduction of Pd(II), but growth is not expected to occur. These results constituted the first report on the capacity of *G. sulfurreducens* regarding palladium reduction which contribute to the knowledge of this strain about its capacity to reduce metals.

On the other hand, methanogenic sludge was tested for its capacity to reduce Pd(II) with favorable results when ethanol was added as electron donor. Anaerobic granular sludge can be described as a spherical biofilm consisting of a densely packed anaerobic microbial consortium with three main groups in there: acidogens, acetogens and methanogens.[4–6] In general, these groups digest the nutrients contained in the waste-water to produce methane as final product. Granular sludge offers excellent settling properties which is ideal for use in UASB reactors, which is technologically appropriate for industrial applications.

In this work key aspects related to the reduction of Pd(II) with the concomitant formation of valuable Pd(0) nanoparticles were studied and the discussion focuses on the feasibility of biogenic Pd(0) production using microorganisms and the

importance of providing an adequate electron donor. Likewise the use of pure cultures versus anaerobic granular sludge is discussed regarding practical applications as well as implications and challenges of the metal recovery technique are discussed in the following sections.

7.2 Importance of operational parameters on Pd(II) reduction and Pd(0) nanoparticles production

a) Electron donor

During this study it was demonstrated that one of the most important aspects to consider for achieving Pd(II) reduction to Pd(0) is the selection of an adequate electron donor (Chapters 2 and 4). The electron donor must sustain and promote interspecies hydrogen transfer or activate metabolic pathways for electron transfer by using the metal as terminal electron acceptor.

Regarding this issue, ethanol was favorably used as electron donor by anaerobic granular sludge to sustain palladium reduction as it was shown in Chapter 4. This substrate offers advantages over other suitable electron donors used in this process (Table 1) since the external supply of H_2 involves high costs and technical difficulties not only to achieve palladium reduction but also to activate it as catalyst.[7] Ethanol as electron-donating substrate is efficient but its success is conditioned to the presence of microorganisms able to ferment it to acetate and H_2, since this reactive hydrogen species promotes the reduction to Pd(0) and its activation. According to this statement it is technologically feasible the application of this scheme since the operation would be simple and less costly since hydrogen will be produced biogenically. Concerning *G. sulfurreducens,* hydrogen and acetate can be used as electron donors, however in order to avoid chemical reactions interference the adequate electron donor to promote only biological Pd reduction is acetate which oxidation can be coupled to the reduction of Pd(II) by using it as terminal electron acceptor (Chapter 2).

G. sulfurreducens oxidized acetate through reactions of the citric acid cycle, and the complete oxidation of acetate to CO_2 releases 8 electrons, which is sufficient to

convert 4 moles of Pd(II) to Pd(0). It means that small quantities of acetate are necessary for Pd reduction and recovery, depending on the concentration of Pd(II) in the influent. For that reason, acetate as electron-donating substrate is a good and feasible option for Pd(0) production by *Geobacter sulfurreducens*.

Moreover the possibility to use the same scheme with other metals of economical importance can be considered by following the main direction of use an adequate electron donor. This process offers not only the recovery of the metal, but also production of valued sub-products, for instance, nano-catalysts.

a) Pd:Cell dry weight (CDW) ratio

The amount of biomass:Pd ratio is a parameter of significant relevance, because it influences two main aspects:

(i) Size of Pd(0) nanoparticles hence, reactivity. Bacteria are a support for Pd(0) NPs since the process of Pd reduction takes place on the cell surface (first step). For this reason, increasing the number of cells respect to Pd concentration provides with more nucleation sites. The formation of more and smaller particles is promoted at high biomass:Pd ratios.[17,18] Moreover, the size of nanoparticles determine their catalytic activity and properties, which can be reflected in high catalytic reactivity towards an specific compound.[17] And as it was demonstrated in Chapter 2, the size of the nanoparticles is a parameter that depends on the surface area that cells offer, hence it depends on the number of bacteria which is a parameter that can be controlled to induce the process as needed.

Table 7.1 Summary of different bacterial species with the capacity of palladium reduction. (Adapted from reference 8)

Species	Electron(s) donor(s) used	Characteristics of interest	O_2 requirements	Reference
Desulfovibrio desulfuricans	Formate Sodium piruvate Hydrogen	Gram (-) Sulfate reducing, metal reducing	Anaerobe	9,10
Desulfovibrio vulgaris	Hydrogen	Gram (-) Sulfate reducing,	Anaerobe	11,12
Shewanella oneidensis	Formate Piruvate Lactate Ethanol	Gram (-) Metal reducing	Facultative anaerobe	13
Cupriavidus necator	Formate	Gram (-) Resistance to heavy metals	Facultative aerobe	14
Rhodobacter sphaeroides	Hydrogen	Gram (-) Photosynthetic, metal resistant	Facultative aerobe	15
Clostridium pasterianum	Glucose	Metal reducing, H_2 producing through fermentation	Anaerobe	16

(ii) Viability of cells. The dose of Pd affects the viability of cells, in Chapter 4 it was demonstrated the inhibition of 50% of methanogenic activity (IC_{50}) of both hydrogenotrophic and acetoclastic groups at concentrations of Pd(II) as low as 0.96 and 2.5 mg L^{-1}, respectively. The amount of cells is a determining factor to achieve Pd reduction, since the metabolic activity is linked to cell viability. This effect was also confirmed by De Windt, et al., 2006, as the viability of *Shewanella oneidensis* was affected at different Pd:CDW ratios.[17]

b) Physicochemical parameters: Pd(II) concentration, solubility, speciation

Metal chemistry and speciation play a major role in the process of metal reduction since it determines its reduction potential and uptake efficiency.[19] The influence of these parameters was deeply studied in Chapter 3. It was demonstrated that speciation is a determining factor, since the formation of solid species depends on the medium pH and on the concentration of Pd(II), which also determines the availability of the metal to bacteria. Indeed, the reduction of solid species is a challenge to cells and in most cases their inaccessibility is limiting to the process.

Other factors, such as the medium composition should be considered, since some kind of compounds can interact or interfere with the metal (for example, sulfur containing compounds), influencing the product.

Recommendations to achieve a process without interferences rely on an adequate pH that guarantees the solubility of metal, a suitable concentration (considering IC_{50} value if viability of cells is required) and avoid the use of sulfur species in the medium composition (to avoid poisoning and formation of solid species).

c) Role of redox mediators

The presence of a redox mediator influences three main aspects of the metal reduction and nanoparticle production: (i) reaction rate, (ii) place of metal nanoparticles deposition in cells and (iii) reduction of solid species.

Redox mediators also referred to as electron shuttles are organic molecules that can reversibly be oxidized and reduced, for that reason they serve as electron carriers among multiple redox reactions.

It has been demonstrated that artificially added redox mediators (that should be membrane permeable) may modify the biological energy metabolism and influence the growth and physiology of organisms.[20] In Chapter 3, it was proven that addition of AQDS promoted faster Pd(II) reduction, formation of extracellular Pd(0) nanoparticles and facilitated the reduction of solid species of Pd such as $Pd(OH)_{2(s)}$. Other studies confirmed the influence of a redox mediator in the reduction of solid species, for instance poorly crystalline iron oxides[21] and in the reduction of metals.[22]

For these reasons, addition of a redox mediator could facilitate the process when adverse conditions are present in the medium, such as solid species or when extracellular nanoparticles formation is induced.

7.3 Mechanisms of Pd(II) reduction: implications of cell-Pd interactions

The mechanisms involved in Pd(II) reduction are complex and include different phenomena, as it was described in Chapters 3 and 4. As it was mentioned above, many factors affect metal chemistry/speciation and consequently the interaction of the metal with binding sites in the cell.

Until now, it has been reported that electrostatic interaction, binding to extracellular polysaccharides and ligand substitution to S, N and P containing sites are involved in the first steps of cell-metal interaction. Once metal-cell interaction is established the metal is close enough to the cell to be reduced. This was called a direct-reduction mechanism, in Chapter 3. However, if a redox mediator is supplemented, the mechanism can be indirect because of the presence of an external substance that facilitates reduction of Pd(II).

In the case of consortia, the metal reduction is supported via reducing agent produced biogenically (bio-H_2). Hence under this scenario extracellular polymeric substances and structure and morphology of the granules are determining factors regarding Pd(0) deposition as it was studied in Chapter 4.

The interaction Pd(0)-cells implies binding to sites containing elements with high affinity to Pd which means that interaction with sulfurous compounds, such as those containing thiol groups of proteins from cells, which could potentially act as inhibiting agent to the catalytic activity.[18] The poisoning of the catalyst can be avoided by using an adequate Pd:biomass ratio.

7.4 Pure cultures versus anaerobic granular sludge for the reduction of Pd(II) and its recovery as Pd(0)

The microbial Pd(II) reduction is of great importance. Until now, different bacteria have been found to be able to reduce Pd(II) to Pd(0) (Table 7.1). Pure strains (*Shewanella oneidensis*[13] and *Desulfovibio desulfuricans*[9]) were the first microorganisms studied and successfully applied to produce Pd nanoparticles.

The interest in some species could be mainly due to their potential and metabolic characteristics, such as metal resistance and extracellular electron transfer, which are desirable in the process of metal reduction. Indeed, in this work the production of Pd nanoparticles by using *G. sulfurreducens* was achieved with excellent results in terms of reaction time and nanoparticles size.

However, for practical applications (pilot or full scale reactor), the use of pure cultures implies some drawbacks related to the sterile conditions required, which is labor-intensive while culturing and handling the bacteria has an economic impact (around €1000 kg^{-1} cell dry weight).[8] Moreover there is an additional cost represented by filtration membranes or encapsulation of Pd nanoparticles in order to prevent leaching of Pd in reactor effluents.

The use of methanogenic granular sludge can avoid those limitations for practical applications since no sterile conditions are needed and the process can be carried out in UASB reactors, which are extensively known and used in many wastewater treatment plants. Consortia offers the advantage of in-situ generation of H_2 necessary for Pd(II) reduction to Pd(0) and subsequent activation as catalyst. Moreover, biogenic-Pd(0) could be associated to granular biomass, which functions as an immobilization method avoinding leaching from the reactor.

Hence, the overall process scheme represented in Figure 7.1 considers a first stage of palladium recovery from Pd(II) containing waste stream or leachate, and its reduction to Pd(0), which is subsequently immobilized in the granule structure and retained in the bioreactor.

In a next step, granular biomass enriched with Pd(0) can be used as a bio-catalyst to transform contaminants using the catalytic properties of Pd to promote different reactions, for instance dehalogenation and hydrogenation.

Under this scenario, reactive hydrogen species are provided in a self-sustained process by fermentation of ethanol. In addition, the concentration of Pd in the influent can be monitored and set in a non-inhibitory value to ensure the microbial activity, which would synergistically act to achieve the removal of palladium and biotransformation of contaminants.

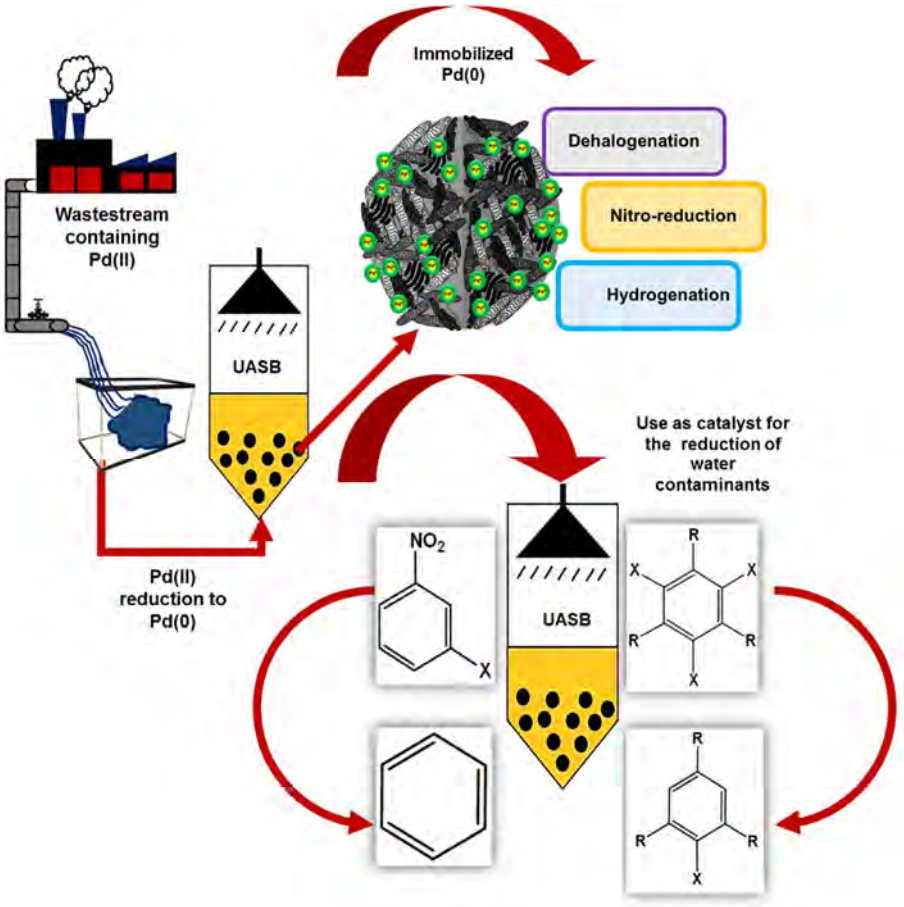


Figure 7.1 Complete scheme of Pd(II) reduction, recovery and immobilization as Pd(0) in granular biomass and its application as catalyst in a biotechnological process to biotransform recalcitrant contaminants such as halo- and nitro- substituted aromatic compounds.

7.5 Implications and challenges of the metal recovery technique: perspectives and future opportunities

Metal recovery from waste streams and biological production of nanoparticles have been demonstrated to be a good and feasible option to replace conventional methods such as solvent extraction, ion exchange, evaporation, cementation and chemical precipitation in order to overcome their shortcomings.

Nevertheless, biological methods have important challenges to become a robust technology. In the next paragraphs the most important points to consider are discussed.

i) *Interference of other metals:* The presence of many metals in the waste stream could cause interference and lead to a decrease in the efficiency of removal or reduction. Since binding to biomass is considered an important step in the mechanism of metal recovery[23,24], metal ions co-existence, in a solution, with equal preference for a binding site would lead to a competition effect between them, lowering the removal/reduction of the targeted element. In other scenario, if metal ion species exhibit preferences for different biomass binding sites, their simultaneous presence in solution may not significantly affect their corresponding metal uptake capacities.[24] For these reasons, it is important to have a deep knowledge about the compounds present in solution; for instance, chemical characteristics, speciation, hydrolysis behavior, stereochemical and the Pearson's reasoning, and in this way challenges can be overcome.

ii) *Biomass as support of nanoparticles in long-term operation*: An advantage of biological methods is that cells represent the available surface area for metal deposition, or in the case of granular biomass, the metal is immobilized within the granules, this offer a support for metallic nanoparticles avoiding their leaching from the system. However, until now there are not studies regarding long-term operation of biological systems

to metal recovery and nanoparticles production, hence there is a need to evaluate the effect of time over the cells or granules as biological supports to determine the life-span of these systems.

iii) *Application of biogenic catalyst and poisoning prevention:* Another important aspect to consider are the characteristics of biogenic Pd(0) synthesized and prevention of its poisoning. Inhibition of the catalytic activity of Pd(0) can be caused for its binding to sulfurous compounds present in biomass, such as thiol groups of proteins, by blocking reactive sites on its surface as it was studied by Sφberger et al.[25] However, this effect of poisoning can be controlled by an adequate biomass:Pd ratio, avoiding or minimizing the use of sulfurous components in the medium and using a consortia or a specific bacterial strain since many studies have reported that bio-supported Pd(0) was more reactive than commercial Pd(0) in different industrial applications[7,7,26,27]. Other alternative is the use of permanganate to oxidize reduced sulfur compounds to prevent poisoning of Pd catalysts which can also be successful in the presence of a large excess of chlorinated organic compounds including chlorinated benzenes.[28]

iv) *Doping of biogenic Pd(0).* This is an area of increasing interest, from both technological and scientific points of view, since bimetallic nanoparticles improve the catalyst quality and properties, and usually incorporate at least one metal from the platinum group which confers relevance to palladium nanoparticles.[29,30] Specifically, for Pd a number of promoting elements can be used, for instance Fe, Cu, Ni, Ag and Au, which could potentially improve either the geometry of the catalyst or the electron transfer efficiency.[31–37] Until now, chemical methods have been used to synthesize bimetallic or Pd-doped nanoparticles; moreover, interactions between the two metals are complex and largely unknown[29], reasons for what there is an opportunity area for future research to prepare bimetallic catalysts with new properties by biological methods. This strategy would

also expand the recovery of valuable metals, such as Rh, Au, Ag and Pt from waste streams.

7.6 Concluding remarks

Until now, only pure strains had been used for palladium reduction, but in the present study it was demonstrated that UASB technology can be successfully applied for palladium recovery with concomitant production of Pd(0) nanoparticles by using anaerobic granular sludge supplied with a suitable electron donor (e.g. ethanol) in a self-sustained method.

The Pd(0)-enriched biomass can be further applied with promising results in the transformation of recalcitrant pollutants, promoting, for instance, reactions of dehalogenation, nitro-reduction and hydrogenation.

Moreover, if the recovery of Pd(0) from the biomass is required, it can be accomplished by simple and feasible techniques such as solvent extraction or burning[25].

Furthermore, inhibitory effects of Pd(II) over methanogenic sludge was evaluated and constitutes the first report on the effect of this metal on microbial communities, which contributes to a better knowledge of the process and a better understanding of variables implied in process control.

The proposed technology for metal recovery and nanoparticles production by using a consortium offers significant advantages over pure cultures, which makes it a feasible and practical option to be applied at full scale.

7.7 References

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