PALLADIUM NANOPARTICLE SYNTHESIS BY S*hewanella oneidensis* MR-1

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Abstract. Metal nanoparticles have multiple uses in industry, technology, medicine, but their production tends to be very expensive and polluting, so scientists are looking into new and greener ways of producing them. One of this eco-friendly method is by using the process of biomineralization done by microorganisms. *Shewanella oneidensis MR-1* is a Gram-negative, facultative anaerobic bacterium that can use a large array of substances as final electron acceptor. When metallic ions are used in the process of respiration as final electron acceptors, metallic nanoparticles may form on the surface of the bacterium. Palladium is one of the many metals that can be used by *S. oneidensis* for the nanoparticles production. Pd nanoparticles have multiple uses, one of its most important utilizations is as a chemical catalyst. In this study, *S. oneidensis* MR-1 was used for the biosynthesis of Pd nanoparticles, as demonstrated by spectrophotometric and fluorometric techniques.

Keywords: biomineralization, nanoparticles, palladium, *Shewanella oneidensis* MR-1.

Rezumat. Sinteza nanoparticulelor de paladiu de către *Shewanella oneidensis* MR-1. Nanoparticulele metalice au utilizări multiple în industrie, tehnologie, medicină, dar producția lor tinde să fie foarte costisitoare și poluantă, de aceea oamenii de știință încearcă să găsească metode noi și nepoluante de a le produce. Una dintre aceste metode ecologice este folosirea procesului de biomineralizare efectuat de către microorganisme. *Shewanella oneidensis* MR-1 este o bacterie Gram-negativă, facultativ anaerobă care poate utiliza o gamă largă de compuși ca acceptor final de electroni. Atunci când ionii metalici sunt utilizați în procesul de respirație ca acceptori finali de electroni, nanoparticule metalice se pot forma pe suprafața bacteriei. Paladiul este unul dintre multele metale care pot fi folosite de *S. oneidensis* pentru producerea de nanoparticule. Nanoparticulele de Pd au multiple utilizări, una dintre cele mai importante fiind cea de catalizator chimic. În acest studiu, *S. oneidensis* MR-1 a fost utilizată pentru biosinteza nanoparticulelor de Pd, fapt demonstrat prin tehnici de spectrofotometrie si fluorimetrie.

Cuvinte cheie: biomineralizare, nanoparticule, paladiu, *Shewanella oneidensis* MR-1.

INTRODUCTION

Nanoparticles (NPs) are particles with sizes between 1 and 100 nm (for at least one dimension) (POTOCNIK, 2011; BATISTA et al., 2015), which have different properties compared with the bulk material. NPs can be classified into natural or synthetic, the latter being produced by either physical, chemical, biological or hybrid methods (JEEVANANDAM et al., 2018). Metal NPs and in special those of noble metals, are of great interest because of their large surface area and their specific function and potential applications (BUZEA et al., 2007; DANIEL & ASTRUC, 2004; HOLT & BARD, 2005; CHEN et al., 2010; AHMAD et al., 2016). Palladium (Pd) is a noble metal from the platinum group metals (PGM) and is one of the most efficient catalysts being intensely studied for its properties (COOKSON, 2012). Pd-NPs play an important role in industry and technology as for example, in the formation of carbon-carbon bound in organic reaction, in the low-temperature reduction of automobile pollutants, in hydrogen storage, electrochemical reactions in fuel cells etc. (KIM et al., 2002; CHEN et al., 2010; SALDAN et al., 2015; SIDDIQI & HUSEN, 2016). The preparation of Pd-NPs can be achieved by different physical, chemical or electrochemical ways (SARTRE et al., 1993; KIM et al., 2003; SON et al., 2004; XIONG et al., 2005a; b; TRISTANY et al., 2006; COOKSON, 2012; ULLAH et al., 2018). However, these methods are very expensive and toxic to the environment due to the use of combustibles, toxic substances, hazardous chemicals, which may pose a biological risk and require lots of energy. The need to develop greener methods that are more energy efficient and environmentally friendly lead the researches into other ways of synthesising nanoparticles such as using organic polymers and different plant extracts (MITOI et al., 2013; KORA & RASTOGI, 2015; SURENDRA et al., 2016). However, true biologically driven production of different metallic nanoparticles is achieved by using different types of living microorganisms (IRAVANI, 2014; MOISESCU et al., 2014; ARDELEAN, 2015; CHEAH et al., 2015). Microorganisms are able to mediate the formation and deposition of minerals directly or indirectly by the so-called process of biomineralization (HEIM, 2011). Microbial biomineralization can be categorised into extracellular or intracellular, depending on the location of the synthesized inorganic materials. For example: *Pseudomonas stutzeri* AG259 can produce Ag-NPs in the periplasmic space as large as 200 nm in diameter (HAEFELI et al., 1984); S*hewanella algae* can produce Au-NPs also in the periplasmic space in anaerobic conditions (NARAYANAN & SAKTHIVEL, 2010); the cyanobacterium *Plectonema boryanum* UTEX485 have been observed to produce Pt-NPs from $PtCl_4$ (LENGKE et al., 2006) and magnetotactic bacteria are known to synthesize Fe-NPs from ferric citrate (MOISESCU et al., 2014). The formation of NPs can even be mediated by viruses, some viral molecules such as fatty acids or amino acids can act as template for the growth of semiconductor nanocrystals (NARAYAN & SAKTHIVEL, 2010). The mechanisms of microbial biomineralization are different, but usually the NPs are formed by trapping the metal ion inside or on the surface of the bacterial cell and then the metal ions are reduced in the presence of enzymes. Another way is by producing organic polymers which can facilitate the nucleation of mineral crystals (LI et al., 2011).

Most metals are toxic to microorganisms (IRAVANI, 2014), therefore microbial resistance to toxic metals is achieved by chemical detoxification, by the energy-dependent ion efflux from the cell through the membrane transport

proteins, and also by the alteration in the solubility of the metal, all playing a key role in their resistance. For example, *Shewanella alge* is able to reduce PCl_6 to Pt-NPs, thus making the media less toxic (LI et al., 2011).

Shewanella oneidensis is a Gram-negative, facultative anaerobe, heterotrophic, gamma-proteobacterium, that can use a large variety of final electron acceptors, such as metal ion, sulphates, nitrates, etc. The ability to reduce heavy metals to an insoluble and less toxic form made this bacterium of great interest for bioremediation studies (XIONG et al., 2005a; b; SURESH et al., 2011).

Shewanella is able to deposit the biogenic nanoparticles that may result after the reduction of metallic ions, in the periplasmic space (KONISHI et al., 2007). Several studies have shown that metal reduction might be achieved by a complex of capping proteins, reductases, cytochromes, quinones, electron shuttles, phytochelatins, known to be able to reduce metal oxides and metals (SURESH et al., 2011).

In the present study we tried to show the ability of *Shewanella oneidensis* MR-1 to produce Pd-NPs by biological reduction of $PdCl_2$ under anaerobic conditions, as a means to develop a recovery method of noble metals through the process of biomineralization.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *Shewanella oneidensis* strain MR-1 (LMG 19005) was purchased from BCCM/LMG Bacteria Collection and pre-grown aerobically in 250 ml Luria–Bertani broth containing yeast extract (5 g/L), sodium chloride (10 g/L), and tryptone (10 g/L). After 24 h of batch inoculation at 30 °C and 150 rpm, the cells in the logarithmic growth phase were harvested by centrifugation at 7500 rpm for 10 minutes, re-suspended in bicarbonate buffer pH 7.0 and re-pelleted by centrifugation. This procedure was repeated twice.

Nanoparticles synthesis. The washed cells biomass corresponding to 2.6 g/L was subsequently re-suspended in 15 ml Falcon tubes filled with 14 mL bicarbonate buffer with 30 mM sodium lactate as the electron donor and 1 ml of 10mM palladium chloride ($PdCl_2$). For the anaerobic conditions, compulsory for nanoparticles synthesis, the culture flasks were sealed after inoculation and anaerobic conditions arose in the medium by oxygen consumption of bacterial cells.

Recovery of nanoparticles from bacterial cells. After 48 hours of incubation, the bacterial biomass with nanoparticles was centrifuged at 7500 rpm, for 10 minutes and then washed two times with MiliQ water. The washed biomass is then heated for 10 minutes at 95°C, in 10% NaOH solution to release the nanoparticles and disintegrate the bacterial biomass. The Pd nanoparticles were pelleted by centrifugation at 14000 rpm for 10 minutes and the black deposit visible on the bottom of the tubes was washed twice with Mili-Q water to remove the NaOH residues.

Analytical methods. The UV-VIS absorption spectrum was analysed with a Specord 210 Plus spectrophotometer (Analytik Jena) and the emission spectra (plasmon) was measured with a FP8300 spectrofluorometer (Jasco).

Dark-filed microscopy and hyperspectral imaging (DMHI). For DMHI, 1 ml of cell suspension was centrifuged at 7500 rpm for 10 minutes washed twice with Sorenson buffer and fixed overnight at 4˚C in 2.5% glutaraldehyde. After fixation, the cells were re-pelleted by centrifugation at 7500 rpm for 10 minutes, washed with deionised water and one drop of the cell suspension was placed on a glass microscope slide, dried at room temperature, and heat fixed. The unstained samples were examined in air and at room temperature by using an enhanced dark field illumination system CytoViva Hyperspectral Microscope (Producer CytoViva, USA) with a 100X oil immersion objective. Spectral data within each pixel of the scanned field of view were captured with a CytoViva spectrophotometer and integrated charged-coupled device (CCD) camera. The spectral resolution was 1.5 nm and the pixel size was 6.45 µm. Spectral data were analysed by using the ENVI 4.8 Image Analysis Software (IDL Available).

RESULTS AND DISCUSSIONS

Palladium nanoparticles biosynthesis. The reduction of Pd(II) to Pd(0) was monitored by colour change of the cell suspension. In Fig. 1a it can be observed the macroscopic aspect of the MR-1 cell suspension in the absence of palladium (left) and after 24 hours of physical contact with the palladium chloride salt (right). After exposure to Pd(II), the colour intensity of the cell suspension gradually turned from pink to light brown (after 24 hours) and then to dark brown (after 72 h), which is a preliminary evidence for the formation of Pd-NPs and reduction of Pd(II) to Pd(0). This experiment also showed that the rate of Pd(II) reduction by the MR-1 cells increases with the increase in contact time. The absorption spectra of both cultures (Fig. 1b) show a change in the Pd(II) 24 hours exposed cells spectra as compared with the non-exposed cells.

UV–visible and fluorometric analysis. The bio-synthesized Pd-NPs were further recovered from bacterial biomass and characterised with the help of UV–vis spectrometry. According to scientific literature, as well as our own experience, the extraction of nanoparticles from cells is needed for improved spectroscopic investigations of these nanoparticles. The absorbance was recorded from 300 to 800 nm. Fig. 2a shows the absorption spectra of Pd-NPs suspension and the absorption of $PdCl_2$ solution, used as a reference sample, for comparison. The 1mM solution of $PdCl_2$ shows a sharp peak at 400 nm, corresponding to the Pd(II) ions in the reference solution. In case of Pd-NPs suspension, the UV–visible spectroscopy shows reduced absorbance spectra (which is characteristic for Pd-NPs) and the disappearance of the peak at 400 nm which indicates the reduction of Pd(II) to Pd(0) (VEISI et al., 2017; SRIRAMULU & SUMATHI, 2018).

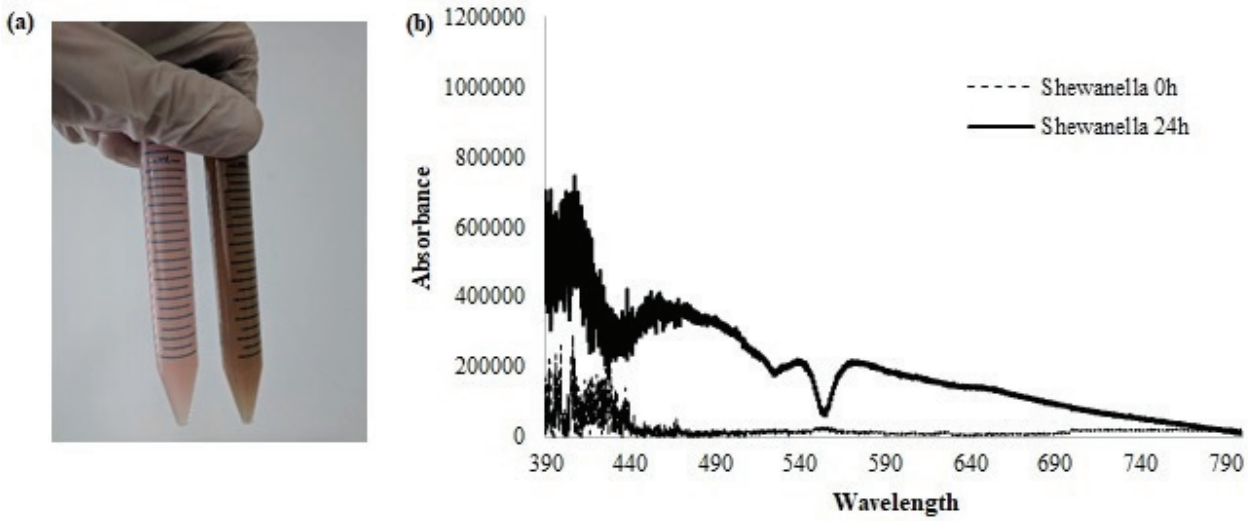

Figure 1. a) The aspect of *S. oneidensis* MR-1 cultivated anaerobically, in the absence (left) and in presence (right) of Pd(II). b) UV-VIS spectra of *S. oneidensis* MR-1 cells cultivated anaerobically in presence of Pd(II), at the beginning (0 hours) and after 24 hours.

Although, in accordance with the available literature (JIANG et al., 2004; NEMAMCHA et al., 2006) a typical absorption spectrum of Pd-NPs solution presents a broad continuous band in the UV-visible range, in our sample a small peak appeared at around 403 nm, that could be assigned to residual Pd(II) from the $PdCl_2$ starting solution.

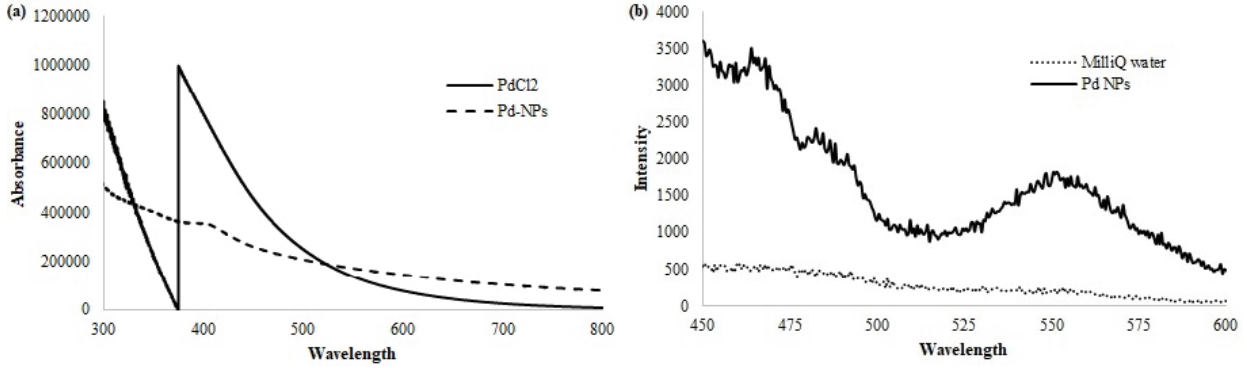

Figure 2. (a) UV-VIS and (b) fluorometric spectra of an aqueous solution of Pd-NPs extracted from *S. oneidensis* MR-1 as compared with the spectrum of $PdCl_2$ solution and deionised water, respectively.

The Pd-NPs suspension thus obtained was further investigated with respect to light emission following specific excitation in UV spectrum (LI et al., 2015; 2017). As one can see the emission spectra of isolated Pd-NPs are clearly different from the matrix (water) in which the nanoparticles are suspended (Fig. 2b), further arguing the different chemical nature of the matrix and of the obtained Pd-NPs through the reduction of palladium chloride by the bacterium *S. oneidensis* MR-1. The solution of $PdCl_2$ in these conditions (e.g. 260 nm voltage of 500V) has a narrow emission peak at 521 nm (98 units in height), completely different (results not shown) in terms of spectrum and intensity from the emission of isolated nanoparticles (Fig. 2b). It has to be noted that chemically synthesized Pd nanoclusters have the maximum excitation wavelength of the 420 nm and the optimal emission wavelength was 500 nm (PENG et al., 2018). Further experiments are needed regarding the size and shape of our reported NPs, in order to better correlate the emission spectrum with these physical details of the NPs produced *in vivo* by *S. oneidensis* MR-1.

Hyperspectral imaging. The CytoViva Hyperspectral Imaging System (HIS) permits the visualization and hyperspectral characterization of nanoscale materials as small as 10 nm, without the need of any fluorescent labelling or pre-treatment of the samples, the nanomaterials appearing brightly lit against a dark background. In the present study we show the results obtained with a hyperspectral enhanced dark-field microscope (HEDFM), consisting of an enhanced dark-field illumination system attached to a standard light microscope, for probing and characterizing biosynthesized Pd-NPs produced by *S. oneidensis* MR-1. Fig. 3a shows a representative hyperspectral image of *S. oneidensis* MR-1 cells exposed to Pd(II) salts. This image reveals the presence of a significant amount of Pd(0) NPs in the cell exterior and associated with cell membrane, identified as bright spots, suggesting that the extracellular and/or membrane-bound proteins play an important role in Pd(II) reduction (NG et al., 2013). These results are consistent with literature data which demonstrates that the preferred localization of Pd-NPs in *S. oneidensis* is in the periplasmic space (NPs <10

nm) or adhere to the surface of the outer membrane facing the extracellular space (NPs ≥ 50 nm) (DE WINDT et al., 2005; KEAT et al., 2015; DUNDAS et al., 2018).

Hyperspectral image analysis was performed on a single, isolated cell and the spectral response was recorded. The spectral profile of the control cells (Fig. 3b) differs substantially compared to the cell with Pd-NPs (Fig. 3c). The control cell profile shows a band with a maximum located at approximately 600 nm while the Pd-NPs cell spectrum has a broader band, with a maximum peak in the range 450-500 nm and a shoulder in the 600 nm region, which was more pronounced in the control cells as compared with the Pd(II) exposed cells, possibly because of the NPs formation. It is interesting to note that the presence of Pd-NPs on the cell membrane induced changes in the peak morphology of *S. oneidensis* cells, revealing a spectral response which combines the spectral features of control and of Pd-NPs cells.

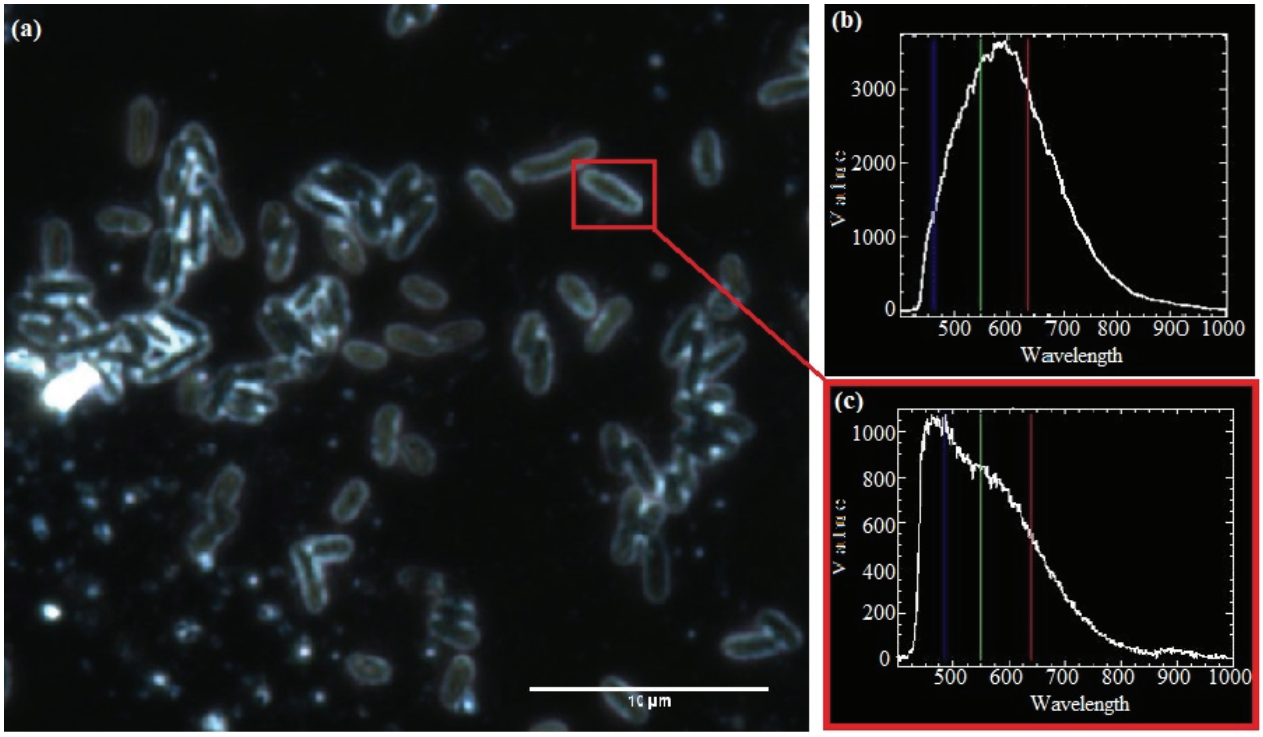

Figure 3. (a) Enhanced darkfield hyperspectral image of bacterial cells with Pd-NPs;
(b) Spectral response of control bacteria and (c) cell with Pd-NPs.

CONCLUSIONS

Our experimental results show, in agreement with the literature, that bacterium S. *oneidensis* MR-1 cultivated anaerobically is able to biologically reduce the Pd(II) from $PdCl_2$ solution to elemental Pd(0) which aggregates and forms nanoparticles. Pd-NPs synthesis verified by various analytical methods such as spectroscopic and microscopic. The Pd-NPs thus synthesized with the bacterium S. *oneidensis* MR-1 can be used as a green alternative to chemical synthesis methods. Therefore, the shape and size and distribution of bio-synthesized Pd-NPs needs to be further investigated using transmission electron microscopy, for their potential use in different applications.

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