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Environmental Remediation

# In vitro evaluation of lead removal in wastewater by *Photobacterium damselae*

## Evaluación in vitro de la remoción de plomo en aguas residuales por *Photobacterium damselae*

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**Abstract**

In order to mitigate the environmental impacts caused by lead in wastewater from sectors such as: mining, petrochemical, metallurgical and others, an in vitro evaluation of lead removal using *Photobacterium damselae* was carried out. Considering isolation and biostimulation phase, obtained isolate was subjected to a selection process in a modified culture medium, to which concentrations of 20 and 100 ppm of Pb were added, finally obtaining the pure strain that showed resistance and /or tolerance to Pb. To determinate the remotionÂŽs capacity of Pb in wastewater two conditions were observed: incubation at controlled temperature (25°C) and incubation at room temperature in Quito-Ecuador (southern zone at 2800msnm). Biochemical characterization of the bacteria was performed using the GN-ID A + B Microgen Kit. In the development of bacterial growth kinetics curves and Pb removal curves, turbidimetry and atomic absorption techniques were used, it was noted that *Photobacterium damselae* presented a greater rate of growth to a maximum of 72 hours and a concentration of 20 ppm in incubation at room temperature achieving a removal rate of 69 % of the lead in the medium. From this information, the potential of this bacterium is inferred and opportunities are opened to continue studies in the future.
***Keywords***: Photobacterium damselae, removal, bioremediation, lead, wastewaters.

**Resumen**

Con la finalidad de mitigar los impactos ambientales ocasionados por plomo en aguas residuales de sectores como: minero, petroquímico, metalúrgico, otros, se realizó una evaluación in vitro de la remoción de plomo utilizando *Photobacterium damselae*. En la fase de aislamiento y bioestimulación las cepas obtenidas fueron sometidas a un proceso de selección en un medio de cultivo modificado, al cual se añadieron concentraciones de 20 y 100 ppm de Pb, obteniendo finalmente la cepa pura que mostró resistencia y/o tolerancia al Pb. La determinación de la capacidad para remover Pb en aguas residuales se observó en dos condiciones: incubación en temperatura controlada (25°C) e incubación en temperatura ambiente de Quito-Ecuador (zona sur a 2 800 msnm). La caracterización bioquímica de la bacteria fue realizada utilizando el Kit de Microgen GN-ID A+B. En la elaboración de las curvas de cinética de crecimiento bacteriano y remoción de Pb, se utilizaron técnicas de turbidimetría y absorción atómica, se destaca que *Photobacterium damselae* presentó una mayor facilidad de crecimiento a un máximo de 72 horas y a una concentración de 20 ppm en incubación a temperatura ambiente lográndose una remoción de hasta el 69% del plomo en el medio. De lo cual se infiere el potencial que tiene esta bacteria y se abren oportunidades para continuar estudios a futuro.
***Palabras claves***: Photobacterium damselae, remoción, bioremediación, plomo, aguas residuales.

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# 1 Introduction

Around the world, soil and water are mainly affected by the extractive industries of metals and non-metallic minerals, which no longer only affect the environment in the form of salts or free elements, but also as part of nanostructures and nanomaterials that bring potential risks to human health and ecosystems (Coccini, Caloni, Ramírez-Cando & De Simone, 2016; Ramirez, 2015), many of them still unknown. Direct consequences include damage to marine and terrestrial ecosystems, which have a negative impact on the economic activities of affected areas (Greenpeace, 2008). In some cases damage to the environment may be considered irreversible; that is, when the damage covers large tracts of land and the remediation is very complicated or costly and considerably affects the animal and plant species, even risking their existence.

The industrial and technological advances of the last decades have caused serious problems of environmental contamination with heavy metals. According to Eróstegui (2009) the main industries responsible for this pollution are mining, petrochemical and metallurgical, among them the most common contaminants are: arsenic, cadmium, chromium and lead (Guevara & Ramírez, 2015) Many of these elements are common in the study of ecotoxicology.

As mentioned by Bustos, Garzon, & Tamayo (2015), the metallurgical sector generates four types of pollution: atmospheric emissions, solid waste, liquid emissions and noise. For authors such as Ambuludi & Hoyos (2013), the generation of solid waste is the one that produces the most pollution, because in the different metallurgical processes heavy metal residues such as lead, nickel, copper, zinc, mercury, arsenic, chromium and cadmium are generated, which can accumulate in the organisms of living beings. And this, indirectly, contaminates sources of groundwater, which are the most difficult to recover.

In order to reduce the public and environmental health problems caused by the different industrial activities, several bioremediation techniques have been used, such as: phytoremediation, which uses plants to concentrate, transfer or destroy contaminants (Volke & Velasco, 2002; Yánez & Bárcenas, 2012), such as Eichhornia crassipes, a perennial aquatic plant widely studied to be used in ex situ phytoremediation, mainly as a tool for the effective cleaning of effluents contaminated with heavy metals, pesticides and dyes discharged by several industries (Guevara & Ramírez , 2015); another technique used to recover and clean these contaminated environments is bacterial remediation, which, according to Sánchez & Rodríguez (2010), is considered as a technology that uses the metabolic potential of microorganisms, specifically their ability to degrade total or partially a wide range of compounds.

Some genera of bacteria such as Pseudomonas, Xanthomonas, Ferroxidans, Ralstonia, Acidobacillus have very interesting capacities as remediating agents, since they are capable of extracting metals from solid substrates, they can be used as bioabsorbents for the recovery of metals and for the treatment of industrial effluents. It is for this reason that the application of these technologies and the subsequent search for continuous improvement become a prevailing need in each of the industrial processes.

The objective of the present study was to evaluate in vitro the ability of Photobacterium damselae (P. damselae) to remove lead in synthetic wastewater, the experiment was carried out under two temperature conditions: incubation at controlled temperature ( 25 ° C) in the Laboratory of the Center for Research and Evaluation of Biodiversity (CIVABI), Southern Campus of the Universidad Politécnica Salesiana and incubation at room temperature (between 5 ° C and 23 ° C for the southern zone of Quito during the study months). Concentrations that exceeded 100 and 500 times the limits considered by Ecuadorian legislation were examined.

# 2 Materials and methods

## 2.1 Isolation and bio-stimulation of the bacteria

The samples of waste water and sediments were obtained from the different effluents and sedimentation lagoons of the mining, petrochemical and metallurgical industries located in Esmeraldas and Zaruma; belonging to the coastal region of Ecuador, using a simple random sampling method. For biostimulation a solution containing: 5g/L of yeast extract, 1g/L of ammonium chloride and 0.1g/L of Morh salt was added. The 1: 1 mixture was allowed to stand between the solution and the sample for 7 days.

For the isolation, a solid medium (Cetrimide Agar) was prepared, then 20 ppm of lead was added, 0.1 and 1 ml of each sample were then inoculated in previously sterilized Petri dishes using the technique of sowing by dipping and striation , And incubated for 72 hours at 25 ° C. The colony forming units (CFU) were then counted and the colonies that had different morphologies were separated. The bacterial isolation was carried out by repetitive seeding of obtained colonies, using the starch depletion technique in a Cetrimida Agar plate, until obtaining boxes with uniform morphology. In order to finish the purity of the isolate, Gram staining was observed microscopically in order to corroborate the uniformity of the strains.

## 2.2 Biochemical characterization of the bacteria

The results of: i) Gram staining; Ii) the oxidase test; And (iii) the biochemical profile, which was obtained using the Microgen GN-ID A + B System kit, according to the user manual (MICROGEN 2015).

## 2.3 Analysis of the kinetics of bacterial growth

The following factors were considered as environmental factors: growth viability, temperature, pH, and colony-forming units as the independent factor. The inoculum was prepared with isolated strains that were able to adapt to concentrations of 20 and 100 ppm Pb in cetrimide medium.

For the preparation of the liquid culture medium, the procedure established by Ramírez & Coba (2012) was taken into account: 1 g of ammonium sulfate, 4 g of yeast extract, 5 g of sucrose, 1 liter with distilled water. And 15 ml of standard Merck lead (1000 ppm) equivalent to 20 ppm of Pb was added. For the second concentration 0.184 g of lead acetate equivalent to 100 ppm of Pb was added. 500 ml of the preparations were then dispensed into each of the flasks by inoculating the strain. A control flask was always provided for each condition, containing the culture medium without the heavy metal and without inoculation of the strain; and duplicate cultures were also counted under negative control.

## 2.4 Elaboration of the calibration curve

McFarland standards were prepared for the preparation of the calibration curve. Once prepared, triplicate readings of each standard and blank or control (distilled water) were taken with the Lovibond Water Testing TB 210 IR turbidimeter. With this data, a linear regression was performed, obtaining the equation that predicts the concentration CFU/ml as a function of the turbidity of the medium over time.

## 2.5 Determination of the removal curve

To determine this curve, liquid culture medium at 20 ppm and at 100 ppm Pb was prepared as previously indicated, the bacterial strain was seeded in duplicate flasks and a sterile flask was taken as reference, incubated at room temperature and at controlled temperature At 25 ° C and the pH and temperature measurements were performed. For the measurement of the Pb concentration, an AA500 Several Atomic Absorption equipment was used, the aliquots were centrifuged at 12g for 1 min. And the concentration of Pb in the supernatant was read. All procedures were performed analogously in the control flask of the experiment.

## 2.6 Determinación de la curva de remoción

Para determinar esta curva se preparó medio de cultivo líquido a 20 ppm y a 100 ppm de Pb, como se indicó previamente, se sembró la cepa bacteriana en matraces por duplicado y se tomó un matraz estéril como referencia, se incubaron a temperatura ambiente y a temperatura controlada a 25°C y se efecturon las mediciones de pH y temperatura. Para la medición de la concentración de Pb se utilizó un equipo de Absorción atómica Varias AA500, las alícuotas fueron centrifugadas a 12g por 1 min. Y se leyó la concentración de Pb en el sobrenadante. Todos los procedimientos fueron realizados análogamente en el matraz control del experimento.

## 2.7 Data analysis

To know if the data followed a normal distribution (pH data and internal temperature of the culture), the Shapiro Wilks Normality test was used, if the test was normal, an ANCOVA model was applied, which allowed to determine if there was or not a difference between the averages of the analyzed data in terms of pH and temperature; and if the result

was not normal the Mann Whitney U test was applied to check if the behavior of the two variables was different, using RStudio and the Stats package. The general charts were made in Excel 2010.

# 3 Results

The isolated strains were characterized using a battery of 25 biochemical tests, which together with the macroscopic and microscopic evaluation showed a taxonomic approximation to P. damselae, with a probability of success of 0.995 (Gramm negative, oxidase positive) according to the identification software of the used Kit (MICROGEN 2015). To determine if this bacterium was able to interact with Pb and not only tolerate the presence of Pb in the medium, we tested: i) two blocks characterized by incubation under controlled conditions at 25 ° C and ambient temperature of the city of Quito (2800 Msnm); Ii) evaluation of the internal temperature of the culture, in order to observe if there is any influence on growth by the examined incubation temperatures; Iii) measuring the pH of the medium during the experiment, so as to show whether there is influence of the same on the growth or, if appropriate, if the pH of the medium was influenced by the concentration of the metal.

When evaluating the capacity of P. damselae, it was determined that the pH is not influenced by the variation of the concentration of Pb, as shown in Figure 1 (A). Extending the analysis shows that there is no difference between the four performed treatments ($p < 0.05$). From this, a pH range (4.5-6.5) is extracted at which the bacterium develops without problems, being exposed to a maximum concentration of 100 ppm of Pb.

Under cultivation conditions, there is no marked difference in the internal temperature between the studied blocks ($p < 0.05$). Always being the lowest ambient temperature and without showing influence by the concentration of Pb as shown in Figure 1 (B). In addition, it showed no significant differences between the ability to remove Pb from the bacteria at different concentrations and temperatures, as shown in Figure 2. In addition, a marked increase was observed in the biomass produced at 25 ° C of the exposed bacteria at 20ppm.

During the 96 hours in which each experiment was individually conducted, it was observed that the incubation at room temperature and 100 ppm represents the highest removal percentage, which was 86 %. However, in terms of absolute values, the reduction occurred up to a concentration of 25 ppm, which is approximately 50 times greater than that allowed by Ecuadorian legislation, the reference value used for the design of the experiment. According to this, the reductions in the blocks at 20ppm achieved a decrease of up to 4 ppm, still reaching a value 10 times higher than the limit allowed for the discharge of wastewater in Ecuador (Figure 3).

# 4 Discussion

Although P. damselae is considered a pathogen in aquaculture (Zorrilla et al., 2003), this is not necessarily a limitation to apply it in closed or batch systems for the recovery of water contaminated with heavy metals. It is well known that many bacteria adapt and can remove heavy metals with some ease (Hasan, Srivastava, & Talat, 2010; Naik & Dubey, 2013; Pongratz & Heumann, 1999). In the present study it was observed that P. damselae was able to remove up to 80 ppm of lead.

Currently there isn't work being done with this bacteria, however similar studies with Aeromonas (Hasan et al., 2010; Paniagua et al., 2006), Pseudomonas (Ramírez & Coba, 2012; Soto et al., 2010) show great capacity of non-enteric bacteria to tolerate, interact and eventually remove or precipitate heavy metals present in black water.

Exploratory studies of this type are an important source of primary information to deepen the knowledge and create a functional database of bacteria capable of removing heavy metals; thus opening lines of research that delve into the mechanism that bacteria use to detoxify contaminated media and/or better understand their molecular characterization in order to know the structure of their genome and classify them functionally in a better way.

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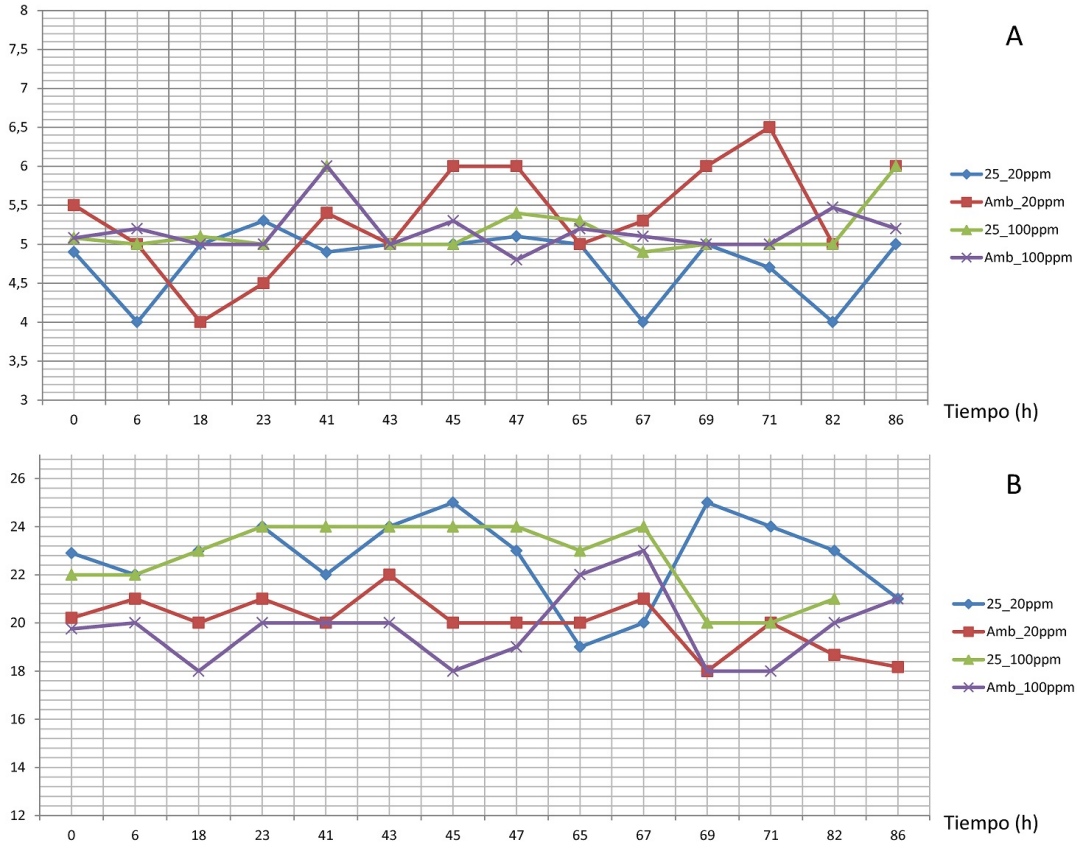


**Figure 1.** Behavior of pH (A) and internal temperature of culture (B) during the study time (96h).

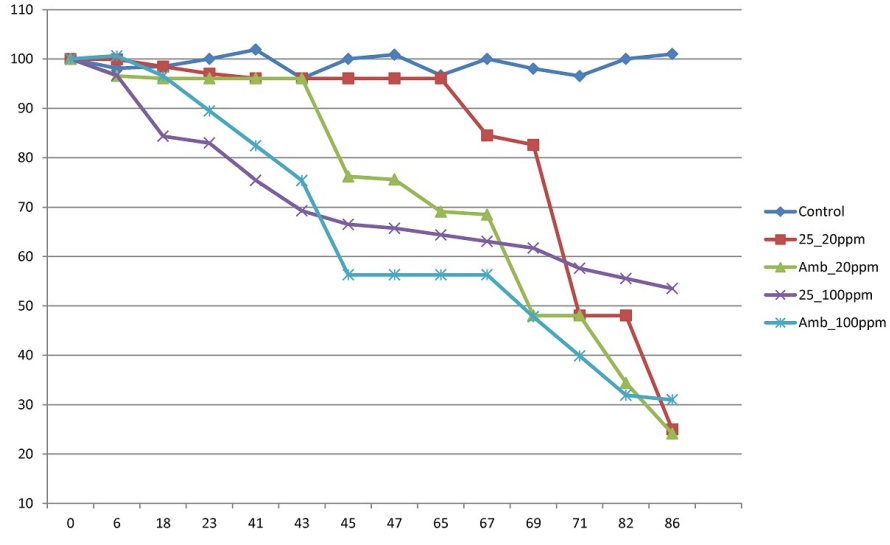


**Figure 2.** Removal Curves *P. damselae* of Pb (%) vs Culture Time

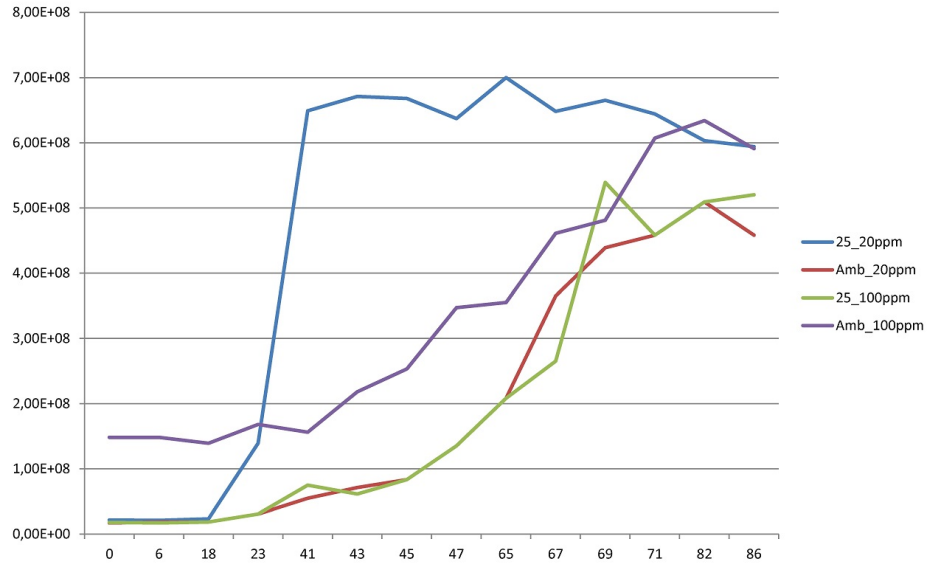


**Figure 3.** Growth curve of *P. damselae* in the study time.

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