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Bacterial selection of the *Pseudomonas* genus with the capacity to treat water and contaminated soils

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Abstract: In the present work, bacteria of the *Pseudomonas* genus native to the Ecuadorian Amazon with the capacity to treat contaminated water and soils were selected. For this purpose, 20 soil samples from Amazon region with evidence of contamination were analysed. For identification, each sample was assigned a code according to the sampling area: Joya de los Sachas (S), Minga (M) and Siete de Julio-Shushufindi (SH). The cultures were performed in the combination of Bushnell Hass (BH) + Luria Bertani (LB) and Müeller-Hinton (MH) + *Brucella* agar (BA) media, all with the addition of diesel to verify their efficacy in the growth of bacteria capable of surviving in contaminated media. The combination with ideal results was that of BH + LB, by means of Gram-staining it was determined that 19 of the samples had interest microorganisms. To characterize the isolates at the species level, biochemical tests of: catalase, citrate, glucose, hemolytic activity and urease were applied, which allowed to confirm the existence of the *Pseudomonas* of interest. The results indicated that *P. stutzeri* (in samples S1 and M1), *P. aeruginosa* (in SH2 and SH5) and *P. putida* (in S7, S8, S10 and SH4) obtaining a total of 8 isolates (40%) of interest from the initial 19. With the results obtained from this work, an optimal culture method was standardized for the selection of bacteria with potential for treating contaminated soils and water.

Keywords: bioremediation, characterisation, contaminated water, contaminated soils, Ecuadorian Amazon, *Pseudomonas* genus

INTRODUCTION

At present, environmental pollution is a subject that has been widely studied, having as one of the main factors the incorrect handling of waste and hazardous materials, polluting both soil and air and water [Kelmendi *et al.* 2018; Martynov *et al.* 2018].

The most common causes of pollution are generally the senseless use of non-biodegradable agricultural fertilisers and pesticides, acid rains, waste from urban and industrial areas in whose composition has a high presence of heavy metals, fats and detergents, there is also the pollution produced due to the extraction and improper handling of oil in producing countries [Ge *et al.* 2018; Wei, Li 2018].

In this sense, oil exploitation in the Ecuadorian Amazon generates a noun of 4.3 mln toxic wastes daily, of which most are discarded into the environment without any prior treatment, in fact, each well that is drilled it produces an average of 4,000 m^3 of waste from within the earth [Barnes *et al.* 2008]. Among the most relevant environmental pollution events in Ecuadorian territory, we find the one that occurred in the Galápagos Islands in 2001, when an unfortunate accident caused the Tanker Jessica, to spill 160,000 gallons of diesel oil and 80,000 gallons of intermediate fuel oil, causing the destruction of the flora and fauna of the area. Other regrettable events are those that happened in Lago Agrio and Shushufindi, which due to the contamination generated by the exploitation and transportation of oil, have caused the death of animals in the area and the emergence of diseases in the native inhabitants of the region [Lagos 2017].

Pollutants such as hydrocarbons, which are put in contact with fertile soil, prevent the exchange of gases with the atmosphere, thus starting a series of simultaneous physical-chemical processes, causing damage in terms of toxicity, extreme salinity, which hinders their treatment, thus destroying the enzymes and biota of the soil [Wasen 2019].

A promising technology for treating contaminated soils and water is bioremediation, which is low cost and does not require the use of sophisticated techniques to result in complete mineralisation. In the same way, it can be carried out in situ, thus preventing the risks that are associated with the transport of contaminated soils [Xu *et al.* 2018]. Bioremediation is a process that occurs naturally in the environment, so it is considered the friendliest method, in fact, the by-products that are generated with this procedure are water, carbon dioxide, and cellular biomass, which are harmless and beneficial to the plant growth process.

The techniques for treating contaminated soils are based on the ability of certain organisms (bacteria, algae, fungi, nematodes and plants) to consume hydrocarbons that will be used as a source of carbon and energy, thus cleaning the contaminated area. Bacteria are the most active and main degrading agents in oil decomposition [Hernández-Ruiz *et al.* 2017]. In this sense, bacteria of the *Pseudomonas* genus present almost in totally natural habitats have been detected, due to the simplicity of their nutritional requirements, in fact, *Pseudomonas* species have been found in environments contaminated with hydrocarbons, showing a bioremediation and biological control activity [Chen *et al.* 2017; Maddela *et al.* 2015].

Pseudomonas is capable of growth using a variety of carbon-rich compounds, including aliphatic, alicyclic, and aromatic compounds found in petroleum [Paulsson *et al.* 2019]. Furthermore, it has been pointed out that *Pseudomonas* may be capable of accumulating polyhydroxyalkanoates (PHAs) from hydrocarbons [Palanivel *et al.* 2020; Prieto 2007].

Among the notable species in bioremediation processes within the genus *Pseudomonas* are: *P. stutzeri*, *P. putida*, *P. aeruginosa* [Palanivel *et al.* 2020].

On the other hand, the most commonly used methods after culture for the identification of these bacteria of interest are: molecular tests, antigen screening, and also biochemical tests, of the latter the most recommended are: catalase test, citrate test, glucose test, hemolytic activity, urease test [Banerjee *et al.* 2017; Guamán *et al.* 2018].

Considering what is rewritten, the objective of this study was to isolate, and identify bacteria of the *Pseudomonas* genus native to the Ecuadorian Amazon with the capacity to treat contaminated water and soils.

MATERIALS AND METHODS

The research was carried out in the laboratories of the Research Department (Molecular Biology Laboratory I and II, Instrumental Analysis Laboratory, and Phytochemical Laboratory) of the State University of Bolívar, for which, 20 samples of contaminated soils of the Amazon were collected (Tab. 1).

For the isolation of bacteria of the *Pseudomonas* genus, a liquid culture in APT medium (buffered peptone water) (64271, Merck KGaA, Germany) of the 20 samples under study was performed, then it was plated on TSA (tryptic soy agar). Subsequently, re-sowings are carried out on a plate as established by Maddela *et al.* [2015]. In parallel, the culture with Müeller–Hinton agar was studied (7101[a], Neogen Corporation, USA) + *Brucella* agar (211086, Becton Dickinson and Company, USA).

Table 1. Experimental material

Origin	Geographical coordinates	Code	Sample quantity
Joya de los Sachas, Orellana	0.3014° S, 76.8571° W	S	10
Minga, Shushufindi, Sucumbíos	0.1794° S, 76.7990° W	M	5
Siete de julio, Shushufindi, Sucumbíos	0.1638° S, 76.7567° W	SH	5

Source: own elaboration.

Crude oil contaminated soil (1 g) was dissolved in 10 cm^3 of sterile 10% saline solution and mixed thoroughly, 2.5 cm^3 of supernatant were transferred to 50 cm^3 of Luria-Bertani broth containing 1% diesel oil and then into Müeller–Hinton and incubated at 37°C for 48 h on an orbital shaker at 100 rpm. To obtain the cell pellet, the broth was centrifuged at 5.000 rpm for 10 min.

The pellet was washed twice with phosphate buffer (pH 6.8; 0.1 M), then dissolved in a small volume of Bushnell Hass (BH) broth medium. Subsequently, 0.1 cm^3 of this suspension was used to inoculate a BH agar plate containing 0.1 cm^3 of diesel oil and on a *Brucella* agar plate. Finally, the plates were kept at 37°C for one week. Pure cultures of bacteria that degrade diesel oil were isolated and stored at –80°C using 25% glycerol.

RESULTS AND DISCUSSION

IDENTIFICATION OF THE OPTIMAL CULTURE MEDIUM FOR ISOLATION OF BACTERIA OF THE *Pseudomonas* GENUS

Colonies that presented a characteristic plate morphology were observed by Gram stain. Characterisation of isolated microorganisms at the species level using biochemical tests. To identify the types of *Pseudomonas* of interest, we performed the following biochemical tests, seeking the following results (Tab. 2).

Table 2. Biochemical characterisation pattern of bio remedial species of the *Pseudomonas* genus

Species	Catalase	Citrate	Glucose	Hemolysis blood β	Urease
P. aeruginosa	+	+	+	+	–
P. stutzeri	+	–	+	–	–
P. putida	+	+	+	–	–

Source: own study.

IDENTIFICATION OF THE OPTIMAL CULTURE MEDIUM FOR ISOLATION OF BACTERIA OF THE *Pseudomonas* GENUS

After the culture on plates, was obtained, as a result, the favourable growth of the bacteria of possible interest in the combination of Bushnell Has + Luria Bertani media, which agrees with the work carried out by Varjani *et al.* [2020] who managed to isolate bacteria of the *Pseudomonas* genus with the use of these culture media. On the other hand, Sepúlveda *et al.* [2014], obtained as a result an adequate growth of *Pseudomonas* with the use of the Müeller–Hinton medium, as well as with *Brucella* agar, however, in the combination of the two media, the growth of the microorganism was null.

INITIAL CONFIRMATION OF ISOLATES BY GRAM STAIN

After culture, by Gram stain, 19 isolates from 20 samples were confirmed as presumptive *Pseudomonas*. For our interpretation, we rely on the article by Danis-Wlodarczyk *et al.* [2016], which tell us that the bacteria of the *Pseudomonas* genus are characterised by being Gram negative.

BIOCHEMICAL TESTS TO THE CHARACTERISATION OF *Pseudomonas* SPECIES

The bacterial strains were resuscitated in Bushnell Hass plates with the addition of diesel and were subjected to biochemical tests, whose results are shown in Table 3.

Table 3. Biochemical characteristics of bacterial isolates

Code	Catalase	Citrate	Glucose	Hemolytic activity (β blood)	Urease
S1	+	–	+	–	–
S2	+	+	–	–	–
S3	+	–	–	–	–
S5	+	–	–	–	–
S6	–	–	+	–	+
S7	+	+	+	–	–
S8	+	+	+	–	–
S9	+	+	–	–	–
S10	+	+	+	–	–
M1	+	–	+	+	+
M2	+	–	+	–	–
M3	+	–	+	–	+
M4	+	+	–	+	–
M5	+	–	–	+	–
SH1	+	+	–	+	–
SH2	+	+	+	+	–
SH3	+	+	–	+	–
SH4	+	+	+	–	–
SH5	+	+	+	+	–

Source: own study.

The catalase, urease and glucose tests allowed us to discard 11 samples (negative catalase, positive urease and negative glucose), which discards them from belonging to the *Pseudomonas* genus and species of interest. The results of the citrate and hemolytic activity tests (β blood) allowed the identification of the remaining *Pseudomonas* isolates, these data were compared and verified with the biochemical test data published by Mays and Manzi [2017] and Meena *et al.* [2020], resulting in the characterisation of *P. stutzeri* (negative citrate and negative hemolysis), *P. aeruginosa* (positive citrate and positive hemolysis) and *P. putida* (positive citrate and negative hemolysis), obtaining a total of 8 isolates (40%) of interest from the initial 19, as evidenced in Table 4.

Table 4. Identification of isolated *Pseudomonas* species

Species	Sample
P. stutzeri	S1, M2
P. aeruginosa	SH2, SH5
P. putida	S7, S8, S10, SH4

Source: own study.

In addition, there are researches in which the bioremediation potential of these *Pseudomonas* species has been determined: based especially on the studies carried out by Palanivel *et al.* [2019], Yuxin *et al.* [2020] and Varjani *et al.* [2020], who managed to significantly reduce the level of contamination, using these bacteria.

CONCLUSIONS

In the current study, bacteria of the *Pseudomonas* genus with the capacity to treat contaminated soil and water were investigated. Twenty soil samples from Amazon region with evidence of contamination were analysed. The results indicated that we were able to determine that the combination that shows the best growth of bacteria with characteristics belonging to *Pseudomonas* genus is that of BH + Luria Bertani medium. What will allow the use of this culture protocol to be able to isolate microorganisms of the *Psudomonas* genus with bioremediating characteristics, prior to this, it would be ideal to carry out *in vitro* tests with this culture medium and, in turn, carry out bioremediation analyses with the isolated strains *in situ*.

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