







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

Henry Joel Escudero-López<sup>1</sup> , Karla Anabel Serrano-Carrillo<sup>2</sup> , Carlos Rodrigo Jácome-Pilco<sup>1</sup> ,  
Herminia del Rosario Sanaguano-Salguero<sup>1</sup> , Isidro Favian Bayas-Morejón<sup>1</sup> ✉ 

<sup>1</sup> State University of Bolívar, Faculty of Agricultural Sciences, Agroindustry Career, Biotechnological Research and Development Center, Km 3 1/2 sector Alpachaca, CP: 020150, Guaranda, Ecuador

<sup>2</sup> San Pedro Educational Unit, Department of Education, Guaranda, Ecuador

RECEIVED 15.10.2021

ACCEPTED 08.03.2022

AVAILABLE ONLINE 29.06.2022

**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 Müller-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<sup>3</sup> 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

**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.

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 Müller-Hinton agar was studied (7101<sup>a</sup>, Neogen Corporation, USA) + *Brucella* agar (211086, Becton Dickinson and Company, USA).

Crude oil contaminated soil (1 g) was dissolved in 10 cm<sup>3</sup> of sterile 10% saline solution and mixed thoroughly, 2.5 cm<sup>3</sup> of supernatant were transferred to 50 cm<sup>3</sup> of Luria-Bertani broth containing 1% diesel oil and then into Müller-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<sup>3</sup> of this suspension was used to inoculate a BH agar plate containing 0.1 cm<sup>3</sup> 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 |
|----------------------|----------|---------|---------|-------------------|--------|
| <i>P. aeruginosa</i> | +        | +       | +       | +                 | -      |
| <i>P. stutzeri</i>   | +        | -       | +       | -                 | -      |
| <i>P. putida</i>     | +        | +       | +       | -                 | -      |

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-WŁODARCZYK *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           |
|----------------------|------------------|
| <i>P. stutzeri</i>   | S1, M2           |
| <i>P. aeruginosa</i> | SH2, SH5         |
| <i>P. putida</i>     | 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 *Pseudomonas* 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*.

## ACKNOWLEDGMENTS

We thank the State University of Bolivar, the Research Department and the debt exchange Project Ecuador–Spain for having made it possible for this investigation to be carried out.

## REFERENCES

BANERJEE S., BATBYAL K., JOARDAR S.N., ISORE D.P., DEY S., SAMANTA I., SAMANTA T.K., MURMU S. 2017. Detection and characterization of pathogenic *Pseudomonas aeruginosa* from bovine subclinical

- mastitis in West Bengal, India. *Veterinary World*. Vol. 10(7) p. 738–742. DOI 10.14202/vetworld.2017.738-742.
- BARNES D., FILLER D., SNAPE I. 2008. Bioremediation of petroleum-hydrocarbons in cold regions. Cambridge University. ISBN 9780511535956 pp. 273. DOI 10.1017/CBO9780511535956.
- CHEN Q., LI J., LIU M., SUN H., BAO M. 2017. Study on the biodegradation of crude oil by free and immobilized bacterial consortium in marine environment. *PLOS ONE*. Vol. 12(3), e0174445. DOI 10.1371/journal.pone.0174445.
- DANIS-WŁODARCZYK K., VANDENHEUVEL D., JANG H.B., BRIERS Y., OLSZAK T., ARABSKI M., ..., DRULIS-KAWA Z. 2016. A proposed integrated approach for the preclinical evaluation of phage therapy in *Pseudomonas* infections. *Scientific Reports*. Vol. 6, 28115. DOI 10.1038/srep28115.
- GE S., ZHU Z., JIANG Y. 2018. Long-term impact of fertilization on soil pH and fertility in an apple production system. *Journal of Soil Science and Plant Nutrition*. Vol. 18(1) p. 282–293. DOI 10.4067/S0718-95162018005001002.
- GUAMÁN J.F., BAYAS-MOREJÓN I.F., ARCOS V., TIGRE-LEÓN A., LUCIO-QUINTANA A., SALAZAR S., GAIBOR-CHAVEZ J., RAMON CURAY R. 2018. Detection of *Helicobacter pylori* from human biological samples (feces) by antigenic screening and culture. *Jundishapur Journal of Microbiology*. Vol. 11(7), e66721. DOI 10.5812/jjm.66721.
- HERNÁNDEZ-RUIZ G.M., ÁLVAREZ-OROZCO N.A., RIOS-OSORIO L.A. 2017. Bioremediación de organofosforados por hongos y bacterias en suelos agrícolas: revisión sistemática [Organophosphorus bioremediation by lice and bacteria in single agrocells: Systematic revision]. *Corpoica Ciencia y Tecnología Agropecuaria*. Vol. 18 (1) p. 139–159. DOI 10.21930/rcta.vol18\_num1\_art:564.
- KELMENDI M., KADRIU S., SADIKU M., ALIU M., SADRIU E., HYSANI S.M. 2018. Assessment of drinking water quality of Kopiliq village in Skenderaj, Kosovo. *Journal of Water and Land Development*. No. 39 (X–XII) p. 61–65. DOI 10.2478/jwld-2018-0059.
- LAGOS P.F. 2017. Petróleo, desarrollo y etnicidad [Oil, development and ethnicity]. *Mundos Plurales-Revista Latinoamericana de Políticas y Acción Pública*. Vol. 4(2) p. 27–27. DOI 10.17141/mundos-plurales.2.2017.3158.
- MADDELA N.R., MASABANDA M., LEIVA-MORA M. 2015. Novel diesel-oil-degrading bacteria and fungi from the Ecuadorian Amazon rainforest. *Water Science and Technology*. Vol. 71(10) p. 1554–1561. DOI 10.2166/wst.2015.142.
- MARTYNOV S., FYLYPCHUK V., ZOSHCHUK V., KUNYTSKYI S., SAFONYK A., PINCHUK O. 2018. Technological model of water contact iron removal. *Journal of Water and Land Development*. No. 39(1) p. 93–99. DOI 10.2478/jwld-2018-0063.
- MAYS J., MANZI L. 2017. Bacterias hidrocarburoclásticas del género *Pseudomonas* en la rizosfera de *Samanea saman* (Jacq.) Merr. [Hydrocarbonoclastic bacteria of the genus *Pseudomonas* in *Samanea saman* (Jacq.) Merr.]. *Revista Colombiana de Biotecnología*. Vol. 19(1) p. 29–37. DOI 10.15446/rev.colomb.biote.v19n1.57408.
- MEENA S., KALAIIVANI V., ABHISHEK D.T., RAMYAA L. 2020. Optimization and characterization of Alginate acid synthesized from a novel strain of *Pseudomonas stutzeri*. *Biotechnology Reports*. Vol. 27, e00517. DOI 10.1016/j.btre.2020.e00517.
- PALANIVEL T.M., NALLUSAMY S., AL-ANSARI A., VICTOR R. 2020. Bioremediation of copper by active cells of *Pseudomonas stutzeri* LA3 isolated from an abandoned copper mine soil. *Journal of Environmental Management*. Vol. 253(1), 109706. DOI 10.1016/j.jenvman.2019.109706.
- PAULSSON M., SU Y.-C., RINGWOOD T., UDDÉN F., RIESBECK K. 2019. *Pseudomonas aeruginosa* uses multiple receptors for adherence to laminin during infection of the respiratory tract and skin wounds. *Scientific Reports*. Vol. 9, 18168. DOI 10.1038/s41598-019-54622-z.
- PRIETO M.A. 2007. From oil to bioplastics, a dream come true? *Journal of Bacteriology*. Vol. 189(2) p. 289–290. DOI 10.1128/JB.01576-06.
- SEPÚLVEDA R.B., HARO I.R., CASTILLO M.S. 2014. Efecto del extracto hidroalcohólico de *Punica granatum* sobre la viabilidad de *Staphylococcus aureus* y *Pseudomonas aeruginosa* “in vitro” [Effect of the hydroalcoholic extract of *Punica granatum* on “in vitro” *Staphylococcus aureus* and *Pseudomonas aeruginosa* viability] [online]. *Revista REBIOLEST*. Vol. 2(1) p. 23–31. [Access 10.09.2021]. Available at: <https://revistas.unitru.edu.pe/index.php/ECCBB/article/view/639>
- VARJANI S., UPASANI V., PANDEY A. 2020. Bioremediation of oily sludge polluted soil employing a novel strain of *Pseudomonas aeruginosa* and phytotoxicity of petroleum hydrocarbons for seed germination. *Science of the Total Environment Journal*. Vol. 737(1), 139766. DOI 10.1016/j.scitotenv.2020.139766.
- WASEN A. 2019. Biodegradation and phytotoxicity of crude oil hydrocarbons in an agricultural soil. *Chilean Journal of Agricultural Research*. Vol. 79(2) p. 266–277. DOI 10.4067/S0718-58392019000200266.
- WEI Y., LI G. 2018. Effect of oil pollution on water characteristics of loessial soil IOP Conference. Ser. Earth and Environmental Science. Vol. 170(3), 032154.
- XU X., LIU W., TIAN S., WANG W., QI Q., JIANG P., GAO X., LI F., LI H., YU H. 2018. Petroleum hydrocarbon-degrading bacteria for the remediation of oil pollution under aerobic conditions: A perspective analysis. *Frontiers in Microbiology*. Vol. 9, 2885. DOI 10.3389/fmicb.2018.02885.
- YUXIN Z., YOU C., FANG Z., JIACHENG W., WEIXIA G., TONG Z., CHAO Y. 2020. Development of an efficient pathway construction strategy for rapid evolution of the biodegradation capacity of *Pseudomonas putida* KT2440 and its application in bioremediation. *Science of The Total Environment*. Vol. 761, 143239. DOI 10.1016/j.scitotenv.2020.143239.