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Isolation of Microorganisms of the Bacillus Genus with Bioremediation Capacity

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ABSTRACT

The objective of this research was to isolate bacteria of the Bacillus genus with bioremediation capacity through the use of selective culture media. The work was carried out in the research laboratory of the State University of Bolivar. Therefore, we started with 20 samples taken from the Amazon and Andean regions in Ecuador where there was contamination. To differentiate each sample, a code was established according to the area where the sample was taken: Guaranda (GD2, GD3, GD4, GD5, GD6, GD7) San Miguel (SM1, SM2, SM3, SP2) Shushufindi (SH1, SH2, SH3, SH4, SH5), and Minga (M1, M2, M3, M4, M5). The culture was carried out in selective media: Luria Bertani for obtaining bacterial cells and Bushnell Hass agar for growth and reproduction, these two media with the addition of 1% diesel. Bacterial isolates were obtained that were analyzed by Gram stain, from this test was possible to verify the existence of Gram + bacteria in 19 of the samples. The isolates were characterized at the species level, using biochemical techniques such as: Catalase, Starch Hydrolysis, Urea, Hemolytic Activity, Nitrate, which helped us to recognize Bacillus cereus bacteria (Sm1, Gda3, Gda4, Gda5, Gda6, Sha1 , Sha2, Sha3, Sha4, Sha5, M1, M2, M3, M4, M5) and β - hemolytic B. thuringiensis, (Sm2, Sm3, Gda2). Concluding that the culture media were specific for the isolation of the Bacillus genus, being able to isolate and characterize 18 out of the 20 samples analyzed.

Key words: Isolation, selective media, Bacillus genus, Bioremediation

INTRODUCTION

In Ecuador, especially in the Amazon region, there is a high degree of contamination due to oil exploitation. Due to oil activity, there have been numerous dangerous events such as liquid and gaseous hydrocarbon spills, in addition to explosions due to pipeline leaks **(Hernández Castellanos, 2016).**

All this deteriorates ecosystems, leaving soils and hydrological basins contaminated, generating atmospheric pollution and all of this results in phenomena such as global warming, climate change and modification of the biogeochemical cycles **(Hernández Castellanos, 2016)**.

Oil spills in water and soil cause irreparable damage to the ecosystem, which requires the immediate taking of preventive and/or corrective measures **(Durval et al., 2019).** In Ecuador, research in the area of heavy metals in soils is scarce, there are no reports or studies that allow us to obtain parameters to define whether an agricultural, residential or industrial soil is contaminated, or if the amount of metals present are within of acceptable levels.

Nowadays, bioremediation has advanced as a viable option for the treatment of soils and water contaminated with hydrocarbons, which, unlike chemical and physical methods, the bioremediation offer many advantages, such as low cost, easy handling and do not generate environmental impact. Bioremediation is a mechanism by which certain microorganisms present in a given place eliminate a contaminant; Over time, the contamination caused by the spillage of hydrocarbons in soils and waters has forced the microorganisms present in them to adapt to the environment in order to survive, degrading the same pollutants, achieving long-term decontamination (**García et al., 2019**).

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There are studies in which the metabolism of bacteria that degrade petroleum hydrocarbons, including: *Bacillus*, *Gordonia*, *Pseudomonas*, *Acinetobacter*, *Mycobacterium*, *Brevibacterium*, and *Burkholderia*, have been analyzed, where, bacteria of the *Bacillus* genus have been classified within the most suitable for biodegradable processes (**Oualha et al., 2019**).

At present, approximately 336 species have been included within this genus, most of these are important for the *B. cereus* group, so the *Bacillus* genus has a main mechanism of action to deal with diseases in plants, through the evolution of biopesticides and Biopesticides, in fact, *B. cereus* and *B Thuringensis* species are related to the bioremediation of water with hydrocarbons. (**Villarreal-Delgado et al., 2018**; **Garzón et al., 2017**).

It is evident that the objective of bioremediation must be the elimination of hydrocarbons, that an adjustment is required in the C, N, and P ratio since the hydrocarbons are composed of carbon. But bacterial activities in the soil may not be appropriate to degrade them to mineralization levels due to the toxicity and complexity of the molecules. Some of the pollutants can be biotransformed and thus become more or less toxic and others become biodegradable, but others cannot be removed from the soil (**Oualha et al., 2019; Merino-Peñafiel et al., 2020)**.

The application of biochemical techniques has made it possible to establish phenotypic relationships and evolutionary relationships between microorganisms, using the morphological description of cells isolated in culture media, with enormous repercussions on bacterial taxonomy, which has given rise to the current classification and identification system. Fast and accurate; among the most notable biochemical tests are: Urease, Catalase, Oxidase, Galactase, Indole, cellulose degradation (**Soto Valenzuela, 2018; Mendoza & Flores, 2017; Cristea, 2016**).

For this reason, the objective of this research was: Identify microorganisms of the *Bacillus* genus for the treatment of contaminated water and soils.

MATERIALS AND METHODS

This research was developed at the Universidad Estatal de Bolívar, Facultad de Ciencias Agropecuarias, Recursos Naturales y del Ambiente, Departamento de Investigación y Vinculación, in the Soil and Molecular Biology laboratories.

Collection Of The Simple

Twenty samples were randomly selected from 2 region in Ecuador (10 from the Amazon región "Shushufindi, Minga" and 10 from the andean region "Guaranda, San Miguel"); distributed as follows: Guaranda (GD2, GD3, GD4, GD5, GD6, GD7) San Miguel (SM1, SM2, SM3, SP2) Shushufindi (SH1, SH2, SH3, SH4, SH5), and Minga (M1, M2, M3, M4, M5). During the collection, 1 g of the soil surface (0–15 cm deep) were obtained from different regions exposed to oil, later the samples were transferred to the laboratory for analysis.

Isolation Of Microorganisms Of The *Bacillus* Genus.

For the isolation of these two species of the *Bacillus* genus (*Bacillus thuringiensis*, *Bacillus cereus*), it was started from a liquid culture in APT "BPW" medium (buffered peptone water). **(EMD MILPORE, VM666728443, Germany)**, then it was plated on TSA Agar (tryptic soy agar): after that, re-plating was carried out as established by **Maddela et al.** (**2015**), where the soil contaminated with 1 g oil was dissolved in 10 mL of sterile saline solution and thoroughly homogenized, then 2.5 of the supernatant was transferred to 50 mL of Luria Bertani broth (TITAN BIOTECH, TM406, India) that contained (1% diesel), it was incubated in an orbital shaker at 37 °C for 48 h at 100 rpm.

To obtain the cell pellet, the broth was centrifuged at 5000 rpm for 10 min, the pellet was washed twice with phosphate buffer (pH 6.8, 0.1M), then it was dissolved in a small volume of Bushnell Hass broth medium (TMMedia, TM053, India) (BH). Subsequently, 0.1 mL of this suspension was used to inoculate a BH + D agar plate.

Finally, the plates are kept in the incubator for 7 days at 37 °C. The pure diesel-degrading bacteria cultures are isolated and stored at -80 °C using 25% glycerol + nutrient broth.

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Initial Confirmation Of The Isolates Obtained

Colonies with characteristic plate morphology were observed by Gram stain microscopy. The strains with specific characteristics to supposedly belong to the *Bacillus* genus presented in the form of a Gram + rod, later, they were frozen in cryovials (10% nutrient broth + glycerin).

Characterization Of Isolated Microorganisms At The Species Level (Presumptive Biochemical Technique For *Bacillus Cereus*, And *Bacillus Thuringiensis*

The presumptive isolates of *Bacillus* cereus were re-cultured following the protocol suggested by **Kramer et al.** (**1982**), which is based on the hemolytic and lecithinase capacity of this bacterium. To this end, serial dilutions were made from the stock solutions. The 1: 1000 dilution was inoculated on blood agar medium (CBA medium).

The colonies that at 24 h of floods presented egg halos. Subsequently, the colonies that, incubated at 30 °C for 24 h, showed hydrolysis halos (lecithinase capacity) were isolated and purified. These colonies were considered as presumptive *B. cereus*.

In summary, for the characterization of *Bacillus* at the species level through biochemical reactions, the following results were sought (Table 1).

Table 1. Biochemical tests for identification of *Bacillus*

Sample (*Bacillus*)	Catalase	Starch hydrolysis	Urea	Blood β	Nitrate
B.Thuringensis	+	+	-	-	+
B. Cereus	+	+	+	+	-

RESULTS AND DISCUSSION

After culturing in the selective media, microorganisms were isolated from each sample analyzed.

By means of Gram staining, 19 of the 20 isolated strains, as shown in figure 1, turned out to be Gram positive, forming short chains, in the same way ellipsoidal and central spores that do not deform the bacillus are observed; given that they have the characteristic bacillary shape of the genus under study according to **Mendez-Ubeda et al.** (**2017**). However, it was required to clearly elucidate between B. cereus and *B. thuringiensis*, for which, Biochemical identification techniques were applied.

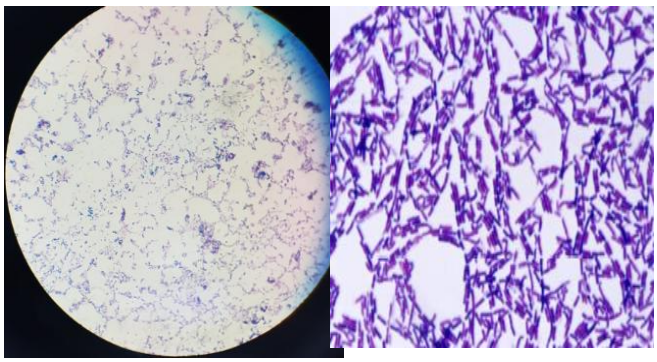

Figure 1. Gram stain in light microscope at 100X

Biochemical tests for the identification of *Bacillus*.

After carrying out the respective biochemical tests on all the isolates, positive (+) and negative (-) results were obtained, which are shown in Table 2.

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Table 2. Results of biochemical tests on

Code	Catalase	Starch hydrolysis	Urea	Blood β	Nitrate
Sm1	+	+	-	+	+
Sm2	+	+	+	-	-
Sm3	+	+	+	-	+
Gda2	+	+	+	-	+
Gda3	+	+	+	+	+
Gda4	+	+	+	+	+
Gda5	+	+	-	+	+
Gda6	+	+	+	+	+
Sha1	+	+	+	+	+
Sha2	+	+	+	+	+
Sha3	+	+	+	+	+
Sha4	+	+	+	+	+
Sha5	+	+	-	+	+
M1	+	+	+	+	+
M2	+	+	+	+	+
M3	+	+	+	+	+
M4	+	+	+	+	+
M5	+	+	+	+	+

bacterial isolates.

Eighteen *Bacillus* isolates were obtained for their respective identification at the species level, the first biochemical test that was performed was the catalase test, in which Gram positive bacilli were observed due to the release of O2; this indicates the presence of the enzyme catalase (Figure 2)

Figure 2. Catalase test.

The identification of the cultures with hydrolytic capacity on starch was carried out by describing the morphological characteristics of the colonies, these colonies being creamy, large, medium, small and with irregular edges; and according to their staining characteristics, they were Gram positive bacilli which are *Bacillus cereus* and *B. thuringiensis* (**Zavaleta, 2018**)

The urea test carried out to know the *Bacillus cereus*, obtained positive results in the following strains (Sm2, Sm3, Gda2, Gda3, Gda4, Gda6, Sha1, Sha2, Sha3, Sha4, M1, M2, M3, M4, M5) the negative strains (Sm1, Gda5, Sha5) were considered to belong to the *Bacillus thuringiensis* species.

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Figure 3. Urea Test.

Later, it was determined that there are hemolytic characteristics, so that *B. cereus* is strongly β-positive hemolytic, (Sm1, Gda3, Gda4, Gda5, Gda6, Sha1, Sha2, Sha3, Sha4, Sha5, M1, M2, M3, M4, M5) *B. thuringiensis* β-negative hemolytic, (Sm2, Sm3, Gda2).

Figure 4. Hemolytic activity test.

Of the 18 isolates of bacteria selected, with this method, 15 isolates with β + hemolytic characteristics, positive catalase (evolution of gas formation of bubbles), urease + reaction were obtained, there was a greenish color change, Gram positive, and 3 of the isolates it was determined that they are *Bacillus thuringiensis*, given that it presented characteristics of β-hemolysis; which allowed locating the two isolates within the Bacillus genus, these data were compared and contrasted with the results of biochemical tests performed **Cortés et al. (2018), y Mendez-Ubeda et al. (2017).**

CONCLUSION

The optimal culture medium for the development and growth of *Bacillus* microorganisms was the combination of luria Bertani and Bushnell Hass media, with results that show more than 80% of isolates specific for the genus of interest.

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