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Isolation and Characterization of Different Bacterial Strains from Soils, With the Potential of Degrading Polychlorinated Biphenyls (PCBs) Under Aerobic Conditions

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***Abstract* - Polychlorinated biphenyl (PCBs) are persistent organic compounds used between 1930 and 1970 as thermal insulators in electrical equipment such as transformers, electrical condensers, heat exchangers, and hydraulic systems. Currently, this xenobiotic is a potential source of pollution to soil and water resources. This research aimed to isolate and characterize bacteria strains possessing a high potential to degrade PCBs contained in used dielectric oils. The bacteria strains were isolated from a soil sample taken from "Alao" Hydroelectric Power Center. Four culture media were used for the soil sample culturing: Agar for Standard Methods Medium (STD), Phosphorus, Ammonium, and Salts Medium (PAS), Minimum Medium (Mm), and Minimum Medium (Mm) supplemented with 25 µL of oil contaminated with 43,6 ppm of PCBs. The isolation, screening, and stabilization process were carried out using three culture media: STD, PAS, and Mm, each supplemented with 25 µL of oil contaminated with 43,6 ppm of PCBs. Nineteen bacterial colonies were isolated and exposed to a higher concentration of PCBs. Eighteen colonies could tolerate 250 µL of oil contaminated with 500 ppm of PCBs. Once stabilized and purified these bacteria strains, were characterized according to their microscopic and macroscopic characteristics and tolerance to heavy metals. Through biochemical tests, 18 strains were identified belonging to *Providencia alcalifaciens, Proteus mirabillis, Providencia rettgeri, Providencia stuartii, Pseudomona alcaligenes, Morganella morganii, and Shigella disenteriae*.**

Keywords: PCBs, Bioremediation, Bacteria, Bacterial Strain, Dielectric Oil.

I. INTRODUCTION

Polychlorinated biphenyl (PCBs) is aromatic organic compounds of carbon atoms, chlorine, and hydrogen. The general formula is C12H10-nCln [1]. PCBs are formed by chlorine substitution in one or more of the ten positions of the biphenyl molecule, resulting in 209 possible combinations of these compounds, called congeners [2]. Among the most common PCBs is Aroclor, a mixture of PCB congeners [3]. Aroclor is obtained through the progressive chlorination of batches of biphenyl until a certain percentage of chlorine is reached [4]

PCBs belong to the group of POPs (Persistent Organic Compounds); they are xenobiotics that are transferred to animals and plants through the soil and water, consequently reaching the highest point of the food chain which is the human being [5][17]; they can also enter the body through the skin and lungs causing skin irritation, hypersecretion of lacrimal glands and conjunctivitis at a chronic level [6].

The electrical sector was the most demanding of dielectric oils containing PCBs as part of its composition, due to the capacity for thermal insulation in generators, circuit breakers, and electrical transformers [2][17]. Currently, installations such as power stations, shunting yards, and hydroelectric plants are being used as storage of contaminated equipment. They are a potential source of pollution to soil and water resources.

There is a characterization of dielectric oils, which details the concentration of PCBs and the equipment from which the oil comes. This characterization is part of the Rational Management Program of oils, equipment, sites, and wastes contaminated with PCBs in Ecuador [7] by the Basel and Stockholm Conventions, where contaminated oils are those with a concentration greater than 50 ppm of PCBs [8]

It is a fact the existence of contaminated oil stored in the country that must be managed and framed in the search for friendly treatments that mitigate the effect of these oils in the surrounding ecosystems to the places where the oils were used and are currently stored.

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Bioremediation uses metabolic processes of living organisms, cellular components, and free enzymes to mineralize or transform a contaminant [9]. Among the studied bacteria strains that degrade PCBs, there are different genera such as *Pseudomonas, Burkholderia, Achromobacter, Comamonas, Ralstonia, Sphingomonas, Acinetobacter, Rhodococcus y Bacillus*[10]. The present study aims to isolate, characterize, and identify bacterial strains that present viable conditions to be considered in a bioremediation process of degradation of dielectric oils contaminated with PCBs.

II. MATERIALS AND METHODS

2.1 Culture Media and Reagents

Dielectric oil contaminated with 43.6 ppm of PCBs, from a circuit breaker located in the "Alao" Hydroelectric Power Plant was used as a selective pressure medium, which was sterilized in an autoclave at 121°C for 15 minutes before the use in the different culture media.

The sample of soil was diluted in peptone water. The microorganisms were cultured for first time using four culture media: (a) Agar for Standard Methods (STD), (b) Minimum Agar Medium of Phosphorus, Ammonium, and Salts (PAS) containing 56,8 g/L K2HPO4; 22,0 g/L KH2PO4; 27,7 g/L NH4Cl; 19,5g/L MgSO4; 5,0 g/L MnSO4*H20; 1,0 g/L FeSO4*7H2O; 0,3 g/L CaCl2* 2H20. The salts were acidified (pH 2.5) with HCl, later 5% (w/v) of yeast extract was added, which provided the source of carbon that the bacterium requires for its survival and development. (c) As a third medium was also used PAS Medium + Contaminated dielectric oil (without the addition of yeast extract) with the intention that bacteria use the oil as a carbon source. (d) The fourth medium was only a minimum medium (Mm) to compare the growth of these plates with the plates of Mm + Oil and analyze the effect of dielectric oil contaminated with PCBs on bacterial growth. The medium PAS, Mm+ oil, and Mm were solidified with 2% (w/v) of agar-agar.

As part of the characterization of bacterial strains, a growth test was performed on metals like As, Cd, Cu, Pb, and Hg contained in Sodium arsenite ($NaAsO_2$), Cadmium nitrate ($Cd(NO_3)_2\ 4H_2O$), Cupric nitrate ($Cu(NO_3)_2\ .3H_2O$), lead acetate ($PbCH_3COO$) and Mercuric nitrate ($Hg(NO_3)$2.H2O).

2.2 Soil sampling

Six sites were selected (up to a depth of approximately 50 cm) where dielectric oil spill stains were observed. A composite soil sample of approximately 1 kg was taken. The sample was stored in a sterilized paper bag and moved in cold and humid conditions to be placed in the laboratory for further study.

2.3 Isolation, screening, and stabilization

Ten grams of the soil sample were dissolved in 90 mL of sterile peptone water, then prepared serial dilution: -2, -3, -4, and -5 in test tubes and allowed to stand for 15 minutes. 25µL of each dilution (inoculum) was spread over the surface of the plate (Petri boxes) prepared with the media described above. All plate cultures were incubated for 24 hours at 30°C. The plate count was done every 24 hours for three days. The colonies obtained were replicated in triplicate in different culture media: STD, PAS, and Mm each one supplemented with 25 µL dielectric oil with PCBs. The bacteria growth was documented for three periods (24, 96, and 168 hours after culturing). Each colony was assigned an Arabic numeration for the screening and those with different morphology were selected for the next step.

To stabilize the isolated colonies, STD, PAS, and Mm plates supplemented with dielectric oil were used, to do this, an inoculum was taken from each colony, and it was distributed in each medium. The bacterial colonies were inoculated three times until a uniform and constant growth is observed.

The colonies were exposed to 43.6ppm of PCBs, contained in dielectric oils, to evaluate their resistance to PCBs. 25 and 100µL of oil were spread separately on plates of STD, PAS, Mm. All plate cultures were incubated for 24 hours at 30°C. The best growing colonies were chosen and exposed to another oil sample, with a concentration of 500ppm PCBs, dosed in volumes of 150 and 250 µL. All plate cultures were incubated for 24 hours at 30°C.

2.4 Culture Purification

The colonies selected in the previous phases were purified by the striating technique, in STD media plates without oil, and incubated for 24 hours at 30 °C. The isolated and purified colonies were taken and re-inoculated in STD plates for further testing.

2.5 Characterization

The bacterial strains were characterized at a macroscopic level by observing and discriminating their shape, elevation, edge, and coloration, and microscopically by Gram staining.

In addition, the resistance of the isolated clones to heavy metals (500µg/mL of As, Cd, Cu, and Pb and 100 µg/mL of Hg) was observed. Biphasic Petri plates were used for replication of each bacterial strain in a culture medium containing a specific metal. On one side of the biphasic plate, PAS media containing heavy metal was used; on another side, STD media was used, as growth control. A single inoculum

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was taken from each colony and inoculated on both sides of the biphasic plate. The plate cultures were incubated for 24 hours at 30 °C.

The growth capacity of bacterial clones at room temperature (17°C) was also evaluated. The colonies were replicated in an STD medium for 48 hours.

2.6 Identification

Biochemical tests identified bacterial strains: oxidase, catalase, citrate, lysine, urease, indole and H2S production, mannitol-mobility fermentation and glucose fermentation, lactose, and H2S production. Biochemical test results were compared with tables that show characteristics and differences between bacterial species to determine the species of each strain in the study [11].

III. RESULTS

3.1 Isolation, screening, and stabilization

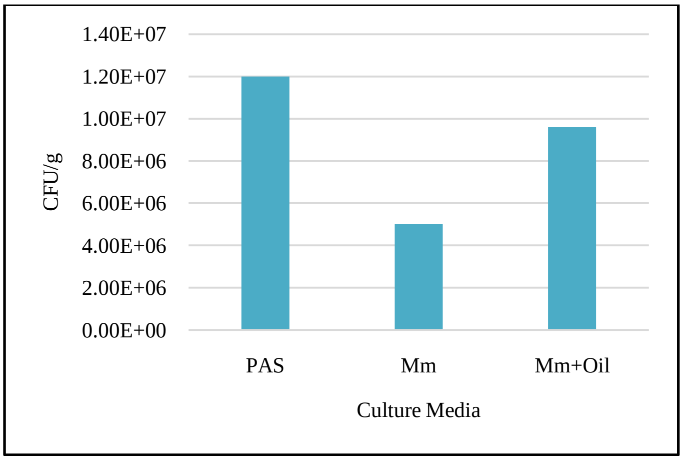

Figure 1: Bacterial growth at 30°C in 24 hours in different culture media

In Figure 1, we can see the results of the plate counts. The cell densities in STD media (about $1,2x10^{11}$ UFC/g) far exceeded the bacterial densities of the others three media: PAS (about $1,2x10^{7}$ UFC/g), Mm (about $5,0\ x10^{6}$ UFC/g) and Mm + oil containing 43.6 ppm of PCBs (about $9,6\ x10^{6}$ UFC/g).

After seven days of planting there were 114 isolated colonies, Table 1 summarizes the isolation of colonies with different morphologies. The colonies that overcame this first process were picked up and transferred to sterile plates as described in the methodology. In the end, a total of 37 colonies succeeded the stabilization process.

Table 1: Colonies Surviving the Isolation Process

Time (hours)	Number of colonies
24	1,13,20,21,23,25,26,27
96	30, 43,44,45, 56, 59,60,66
168	72, 78-82, 84-87, 89, 92-94, 100, 102, 103, 105,106,110,114

For stabilization, as expected, all colonies grew at the first inoculation in STD media, and they were transferred to sterile culture media plates. Colony number 78 took longer to grow compared to the other colonies; colony number 106 grew only in the STD medium, and many colonies did not grow on any plates. Most of them presented better growth in STD media. However, some colonies grew in the three media, and others such as colonies 100 and 105 grew better in the Mm media containing oil than in the STD medium, therefore, demonstrating their ability to grow at the expense of oil contaminated with PCBs. From this process 28 colonies were viable. In the next step, the viable colonies were exposed to two doses of dielectric oil containing PCBs to evaluate their resistance.

At 43.6 ppm PCBs, dosed in volumes of 25 and 100 µL at a temperature of 30°C, the colonies that perished were the number 13, 81, 86, 87, 89, 93, 94, 102, and 114. The 19 colonies resistant to the previous concentration were exposed to a concentration greater than 500 ppm of PCBs (in volumes of 150 and 250 µL). In this step, colony number 60 perished. Plates with 250 µL were practically flooded with oil contaminated with PCBs, even so, all colonies were able to grow in at least one of the plates, adapting or possibly metabolizing the PCBs contained in the oil. Our report indicated that colonies 25, 84, 1, and 30 grew better in the culture plates with 150 µL of oil added. Colony 82 survived surrounded by oil (250 µL) but only in the plate with STD medium, while colonies numbers 92 and 60 failed to survive this condition. Colonies 60, 44, 25, 92, 30, 84, 1, and 82 did not grow in Mm media added with 250 µL of oil, being ineligible to assess their ability to degrade PCBs. Finally, eighteen colonies with a high potential to degrade PCBs were obtained.

3.2 Characterization

The 18 colonies were characterized macroscopically by their morphology, predominating a circular shape, convex elevation, integer margin, and internal cream color; microscopically all colonies were identified as Gram-Negative Bacilli.

Regarding the resistance to heavy metals, the test was performed in a PAS medium supplemented with five heavy metals. All the isolated strains were able to grow in at least

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one of the tests. In the medium supplemented with cadmium, 83% of the total clones were obtained (Figure 2), followed by the culture plate containing mercury with 78%. While arsenic with 44% growth turned out to be the most restrictive heavy metal for developing strains.

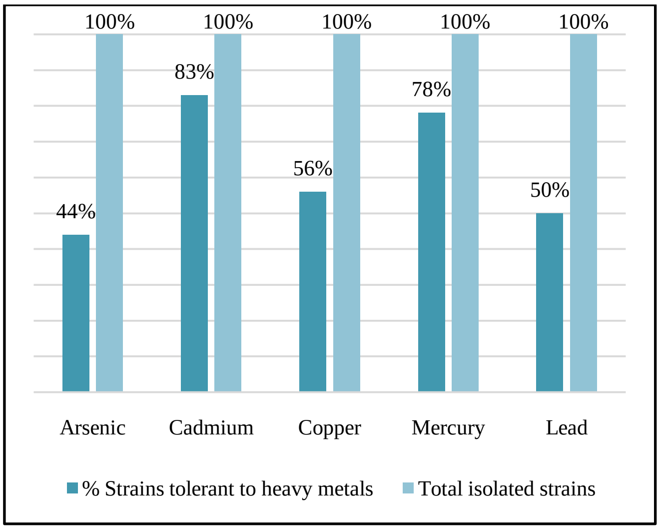

Figure 2: Percentages of the total isolated bacteria strains resistant to heavy metals

Isolated strains were also characterized by growth at room temperature. The ambient temperature did not restrict the growth of any colony in the STD media; however, it took a long time to grow (48 hours) than the conditions used in this study (24 hours at 30 ºC). Colonies 44, 79, 45, 72, and 85 of the plate with PAS media did not grow within 48 hours. In the Mm media, few colonies grew (among them 100, 105, 26, and 72), however, under this criterion, these colonies demonstrated the best adaptation to grow with minimal nutrients and at room temperature.

3.3 Identification

The genus and species of the bacteria strains were determined. Seven bacterial strains were identified: Providencia alcalifaciens, Proteus mirabillis, Providencia rettgeri, Providencia stuartii, Pseudomona alcaligenes, Morganella morganii, Shigella disenteriae.

IV. DISCUSSION

All isolated clones showed growth in Mm media (Figure 1). However, in the medium Mm + oil was observed with higher growth (nearly double) compared to the Mm culture plate. It demonstrates that dielectric oil contaminated with PCBs confers a growth advantage to some bacteria and could even be using it as a carbon source. This observation is confirmed by the result obtained in the PAS medium culture plate growth, which is similar to the observed in the Mm + oil contaminated with PCBs media culture plate, between both media the only difference is the carbon source. The PAS medium contains yeast extract which functions as an alternative source of carbon and energy for bacterial growth [12]. In contrast, the carbon source of Mm + oil media is the oil contaminated with PCBs, which are more challenging to digest than the yeast extract. Therefore, the slight variation in growth, in favor of the PAS medium, is explained because the carbon of yeast extract is faster and easier to obtain and digest by bacteria than the carbon contained in the oil and PCBs [13].

The lower bacterial growth observed in plates containing only Mm media could be caused by the low concentration of nutrients or by the requirement of a longer incubation time. To rule out this assumption, after plate counting, the plates were left in incubation at 30º C for 7 more days. However, any significant variation was not found.

Due to the presence of heavy metals in mineral oils [14], it was decided to evaluate the tolerance to heavy metals of the isolated strains and consequently enhance the subsequent study of these species for future applications in techniques of bioremediation. At the same time, a selection criterion related to the ability of bacteria to survive in hostile media similar to dielectric oil with PCBs was applied to the selected strains.

Lead, mercury, arsenic, copper, and cadmium are heavy metals commonly released in various industries, becoming highly toxic and dangerous compounds for health and the environment [15]. Many Gram-Negative bacteria have been studied to test their resistance to various heavy metals, demonstrating that a strain may be able to resist downright hostile means and still survive [16].

V. CONCLUSIONS

Bacterial strains were isolated from soil from the "Alao" Hydroelectric Power Plant of Electricity Company Riobamba S.A. They were characterized by adapting parameters to PCBs, resistance to different concentrations of dielectric oil contaminated with PCBs, macroscopic and microscopic characteristics, resistance to heavy metals, and growth capacity at room temperature.

Using four media: Agar for standard methods (STD), PAS, Minimum Medium (Mm), and Mm added oil containing PCBs, 18 bacterial strains were isolated under selective pressure and increasing concentrations of PCBs contained in dielectric oil.

Through the application of biochemical tests, 18 bacterial strains were identified. These strains corresponding to the species Providencia alcalifaciens, Proteus mirabillis, Providencia rettgeri, Providencia stuartii, Pseudomona alcaligenes, Morganella morganii, Shigella disenteri.

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Since this is a new study at the national level it is suggested to continue the research to seek to extrapolate the conditions of the in vitro test to a pilot plant, controlling environmental factors, as well as stabilizing the cell growth of the strains found to achieve higher rates of growth.

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