Selective Isolation and Phenotypic Characterization of Bacteria and Actinomycetes from Oil-contaminated Soils

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Abstract. Large numbers of bacteria and actinomycete strains were isolated from two contaminated soils with high total petroleum hydrocarbons (TPH) content. From five selective media formulations used, nutrient agar and glucose yeast extract agar showed the best results. Bacterial colony forming units per dry gram soil (ufc/g) ranged from 5.14x10³ to 4.20x10¹¹, whereas for actinomycetes was from 8.99x10⁴ to 9.2x10⁷ ufc/g. Seventy-three bacteria and ninety-three actinomycete isolates were recovered from the two soils. Numerical taxonomy analysis of phenotypic data shows that at 90% similarity, the bacteria could be divided into sixteen multimembered and fifteen single-membered phenetic-groups, while the actinomycetes were separated in twenty multimembered and four single-membered phenetic-groups. Out of the one hundred and sixty-six strains, only ten were unable to use gasoline or oil as sole carbon and nitrogen sources. Most of the isolates could degrade both hydrocarbons. The results of this study show that bacteria and actinomycetes are present in large numbers in high TPH contaminated soils, with an extraordinary degree of diversity and that they can use oil or its derivatives as nutrients.

1. Introduction

Oil production around the world is a process that involves many steps where contamination risks to the environment are potentially high [1]–[3]. Efforts to clean the environment of pollutants that come from the petroleum industry have been implemented in oil-producing countries [4]. Modern techniques are used to clean water or soils where oil spillages have occurred. These combine chemical, physical and biological methods with varied rates of decontamination success [5]–[7]. Many biological techniques rely on microorganisms that come from different bacterial genera such as Acinetobacter, Alcanivorax, Gordonia, Pseudomonas or Rhodococcus [8],[9]. These bacteria have been isolated from diverse environmental sources, where soils with some content of hydrocarbon contaminants from oil have been the preferred choice [10],[11].

Many microbial groups are better suited to adapt to petroleum contaminants and its many derivatives in the soil [12],[13]. Dependant culture methods have been looking for that type of microbes, which are very common in contaminated soils. These actinomycete, bacteria and fungi grow very quickly on rich nutrient media. Members of Proteobacteria, and a few from the class Actinobacteria have been isolated and characterised, with some of them used commercially in bioremediation programs [11],[13]–[15]. On the other hand, molecular techniques have shown that there is a sizeable microbial population that has not been studied. These microbes belong to bacterial groups that are challenging to
isolate in pure culture. One alternative to get growing these types of microorganisms could be by developing new approaches to look for potentially novel bacterial species capable of transforming oil pollutants into harmless compounds [10],[15],[16]. This study aimed to isolate and characterise bacteria and actinomycetes present in oil-contaminated soils and to assess their potential to use hydrocarbons sources as nutrients.

2. Materials and Methods

2.1. Soil Samples
Two soil samples were provided by LAPSU S.A. (an Ecuadorian private laboratory that performs chemical analysis for the oil industry) which had been collected from the Sacha Oil Fields in the Ecuadorian Amazon Basin. Soil sample A contained 31000 ppm of total polycyclic hydrocarbons (TPHs) and a high content of clay. Sample B was a sandy soil with 46000 ppm TPH content. Both samples were used to carry out the process of selective isolation of any bacteria and actinomycete strains that had been present in the soils. Once the samples arrived at our facilities, they were homogenised with the help of a sterile mortar and pestle and kept at a temperature of 10°C in sterile plastic flasks.

2.2. Moisture and Organic Matter Contents.
Five grams of each soil sample was added to a porcelain crucible and dried at 105°C for 24 hours. This procedure was performed in triplicates and the moisture content recorded as the average of weight loss to the initial weighed soil. A known sample of the dried soil was weighed and placed into a new crucible and placed in a muffle furnace at 400°C for 24 hours, to burn any organic matter present in the samples. Before the crucibles were weighed again, they were cooled down to room temperature. The organic matter content was determined as the percentage of the weight loss in each of the triplicates.

2.3. Electrical Conductivity and pH of the Soil Samples
Ten grams of each soil were placed in 100 ml beakers where 25 ml of distilled water was added. The resultant soil suspension was stirred for 30 minutes. A thin aqueous layer was visible on top of the soil suspensions after the samples were left to equilibrate for one hour. The electrical conductivity and pH values were obtained using a glass electrode pH meter (Fisher Scientific, Acumet AB200), once the reading in the equipment stabilised. The final results were reported as the average of three recordings per sample.

2.4. Selective Isolation of Microorganisms
Before the selective isolation of any bacterial and actinomycete strains present in the samples, the colony forming units per dry weight soil and colony diversity were determined. Ten grams of each soil was placed in a glass bottle that contained 90 ml of sterile tap water. For the bacterial studies, serial dilutions were prepared down to 10⁻¹ and 100 uL from dilutions 10⁻² to 10⁻⁴ were spread over the surface of two sets of sterile media that had been added either gasoline or crude oil. The basal medium contained 1ml of trace salts solution (MgSO₄.7H₂O, FeSO₄.4H₂O, CuSO₄.4H₂O, each at a concentration of 0.1g/100/mL), K₂HPO₄ (4 g/L), KNO₃ (2 g/L) and agar (15 g/L). Additionally, 100 uL from dilutions 10⁻⁴ to 10⁻⁷ were inoculated over the surface of nutrient agar (NA), glucose yeast extract agar (GYM) and glucose yeast extract malt extract agar (GYEA). For the actinomycete selective isolation, the dilution 10⁻² was prepared separately and placed in a water bath at 55°C for 6 minutes. The dilutions 10⁻³ and 10⁻⁴ were prepared, and 100 uL of these serial dilutions were spread over the surface of plates that contained starch casein agar (SCA) and glucose yeast extract agar (GYM). All plates containing the different media formulations were supplemented with nystatin (75 ug/mL) and adjusted to a pH of 6.5. After the inoculation procedure was completed, the plates were left to dry at room temperature for half an hour and then incubated at 26°C, one week for bacteria and two weeks for the actinomycetes. The colonies growing on the surface of the many media were expressed as colony forming units per dry weight soil and as the number of different colonies based on their macro-morphology.
2.5. **Isolation, Purification, and Preservation of the Strains**

Seventy-three bacterial and ninety-three actinomycete colonies were isolated using sterile toothpicks on NA or GYM, respectively and incubated at 26°C. The resultant microbial cultures were examined by eye to discard any contamination. All pure cultures were used to inoculate fresh agar plates and to prepare cell or spore suspensions in 20% glycerol (w/v) for long-term preservation and 1 ml sterile tap water tubes for the phenotypic tests.

2.6. **Colour Grouping of the Isolates**

Microbial cultures that were grown on AN and GYM plates were used to determine the colony pigmentation of the bacterial cultures whereas for actinomycete strains the colour of the aerial spore mass, substrate mycelium and any diffusible pigments. In both cases, a standard colour chart from the British Standard Specification for Colours for Identification Coding and Special Purposes was used. Strains that shared the same pigments were grouped to form preliminary bacterial and actinomycete colour groups.

2.7. **Microscopic Characterization**

Bacterial smears on microscopic slides were prepared to determine cell shape, Gram stain, and any capsule or endospore formation. Gram stain was achieved by covering a fixed bacterial smear with crystal violet for 1 minute then rinsed with distilled water. Then, an iodine colourant was used for 1 minute and washed with 100% ethanol. Finally, safranin was used as a counterstaining dye for 30 seconds. Capsule determination was performed by using a non-fixed bacterial smear which was stained with crystal violet for 1 minute and washed with a 20% copper sulphate solution (w/v). Finally, any bacterial culture that presented gram-positive bacilli characteristics was used to determine the presence of endospores inside its cell. To that aim, a fixed bacterial smear was stained with malachite green for 10 minutes, in a hot water vapour atmosphere. Safranin was used a counterstaining dye. At the end of all these techniques, the glass slides were left to dry at room temperature prior observation under a binocular microscope. For actinomycete cultures, sterile coverslips were inserted at an angle of approximately 45°, in GYM plates that had been previously streaked with a fresh actinomycete inoculum. After a 10–12 day incubation period, the coverslips we taken out of each plate and fixed on the surface of a glass slide. With the help of a light microscope, the spore chain morphology was determined for each actinomycete strain.

2.8. **Physiological Tests for Growth**

Five microliters of the cell or spore aqueous suspensions were used to inoculate plates with different media formulations and incubation conditions. Temperature, pH, and NaCl tolerance range growth were determined for the whole bacterial and actinomycete culture collection. As otherwise stated in each test, bacterial strains were inoculated on nutrient agar whereas actinomycetes were on GYM agar. After the incubation period, the presence of actinomycete or bacterial growth was recorded as 1, and absence as 0. Table 1 shows the details of each test.

| Test          | Conditions                        |
|---------------|-----------------------------------|
| Temperature   | 4°C, 37°C and 50°C                |
| pH (buffered) | 4.5, 5.5, 6.5, 7.5 and 8.5        |
| NaCl (w/v)    | 1%, 5% and 10%                    |

2.9. **Determination of the Ability to Use Hydrocarbon Compounds as Sole Nitrogen and Carbon Sources**

A basal medium (1ml of trace salts solution, K₂HPO₄, 4 g/L, and agar, 15g/L) supplemented with crude oil or gasoline (5 mL per litre), was used to determine the ability of the bacterial and actinomycete strains to grow on these two hydrocarbon compounds. The pH of the media was adjusted to pH 6.5. Five microliters of the bacterial or spore aqueous suspensions were inoculated over the
surface of the sterile culture media. Plates were incubated at 26°C for two weeks, and the presence of growth was recorded.

2.10. Numerical Taxonomy of Phenotypic Data
All the results from the phenotypic characterisation were used to construct a data matrix in binary code. The data were analysed with the NTSYS programme suite [17]. Similarities were calculated using the simple matching coefficient (Ssm) and Jaccard’s coefficient (SJ). Clustering was achieved by the UPGMA (Unweighted Pair Group Method with Arithmetic Means) algorithm. Phenetic clusters were identified by analysing the results from the two resulting dendrograms.

3. Results and Discussion

3.1. Physicochemical Properties of the Samples
Table 2 shows the results for the physicochemical characteristics of the soil samples. The high amount of TPHs in soil sample B is probably responsible for the higher counts on electrical conductivity, organic matter content, and pH.

| Parameter                      | Sample A | Sample B |
|-------------------------------|----------|----------|
| Main soil particle            | Clay     | Sand     |
| TPHs (ppm)                    | 31000    | 46000    |
| pH                            | 4.99     | 6.37     |
| Electrical conductivity (uS/cm)| 127      | 5190     |
| Moisture Content (%)          | 29.96    | 4.11     |
| Organic Matter Content (%)    | 18.10    | 35.02    |

3.2. Number and Colony Diversity of Bacteria and Actinomycetes
The colony forming units per dry weight soil and colony diversity are shown in Table 3. The results from the isolation medium that contained crude oil are not shown because it was challenging to differentiate the colony growing on the dark surface of the medium and fungal growth from strains that apparently are resistant to the nistatyn concentration used in the medium.

| Soil Sample | Selective medium | Colony Forming Units per Dry Weight Soil | Different Colony Number |
|-------------|------------------|-----------------------------------------|-------------------------|
| A           | AN               | 1.55E+09                                | 14                      |
|             | AG               | 2.47E+07                                | 5                       |
|             | GYM              | 5.14E+03                                | 3                       |
|             | GYEA             | 1.35E+04                                | 6                       |
|             | AN               | 4.20E+11                                | 6                       |
|             | AG               | 1.36E+09                                | 11                      |
|             | GYM              | 1.29E+08                                | 5                       |
|             | GYEA             | 1.50E+08                                | 4                       |

In general, more significant numbers of bacteria were found in sandy soil B, despite that the TPH value is close to 1.5 times higher than in sample A. It seems that the sandy soil texture makes more oxygen available, giving an opportunity to the microbes present to adapt better to the conditions [18]. The results also show that the electrical conductivity and TPHs content do not affect the microbial population. Another factor for lower cfu counts found in soil A could be attributed to the pH of de soils and the pH of the selective media. The pH in sample B is very close to the one used in this experiment whereas the pH in soil sample A is acid (4.99). There is a chance that the microbes present
in soil A were not able to grow due to the almost neutral pH on the selective isolation plates. Regarding the different bacterial colonies growing on the plates, there are no significant differences when comparing the two soils. On the other hand, when analysing if there is any correlation between the cfu counts and the selective media used, it is evident that there are higher numbers related to nutrient agar than the rest of the media. It is well known that NA is a nutrient rich medium that has significant amounts of compounds quickly available for microbial use. In the other selective media, it is probable that many of their components are not available unless the microbes possess the enzymatic machinery to use them.

For the actinomycete count, Table 4 shows the colony forming units per dry weight soil and colony diversity. The results agree to what was found in the bacterial population. Soil sample B presents higher actinomycete cfu/g numbers than soil sample A, however, the differences in numbers are not as significant than in the bacteria. What it is remarkable it is that regardless of soil sample or isolation media, the actinomycete counts are very high for this type of contaminated soils. This kind of numbers is typically found in non-contaminated soils [19],[20]. For us, this is the first report of such findings, probably meaning that actinomycetes could play a significant role in bioremediation programmes. It is widely known that nocardioform actinomycetes can degrade different hydrocarbon sources. However, not much is known about what other non-nocardioform actinomycetes can do in such circumstances.

**Table 4. Colony forming units per dry weight soil and colony diversity of actinomycetes.**

| Soil Sample | Selective medium | Colony Forming Units per Dry Weight Soil | Different Colony Number |
|-------------|------------------|------------------------------------------|-------------------------|
| A           | GYM              | 7.04E+07                                  | 16                      |
|             | SCA              | 9.20E+07                                  | 11                      |
| B           | GYM              | 8.99E+04                                  | 6                       |
|             | SCA              | 2.70E+06                                  | 5                       |

3.3. **Colour Grouping of Bacterial and Actinomycete Strains**

Seventy-three bacteria and ninety-three actinomycete strains were isolated from both soils samples. Table 5 and 6 show the corresponding grouping by colour similarities between the different bacterial and actinomycete isolates. Sixteen multimembered and six single membered colour groups were recovered for the bacterial collection, whereas fifteen multimembered and thirteen single membered colour groups for the actinomycete isolates. These differences could be because of more actinomycete strains were isolated from the contaminated soils, and in any case, higher colony diversity was found on the media plates that corresponded to the actinomycetes.

3.4. **Phenotypic Characterisation of the Isolates**

Figures 1 and 2 show the dendrograms from the analyses of the different phenotypic tests carried out for the bacterial and actinomycete isolates. The dendrograms were drawn based on similarity matrices calculated from datasets consisting of 40 and 53 results reported as binary code, from the phenotypic characterisation of the bacterial and actinomycete isolates. The bacteria could be divided into sixteen multimembered and fifteen single-membered phenetic-groups, while the actinomycetes were separated in twenty multimembered and four single-membered phenetic-groups.
Table 5. Assignment of bacterial isolates to multimembered and single-membered colour-groups.

| Colour Group | Colony Colour | ID Actinomycete Isolates |
|--------------|--------------|--------------------------|
| 1            | Yellow       | TG17, TG24, TG25, TG26, TG27, TG61, TG62, TG82, TG83, TG84, TG89, TG92, TG119, TG120, TG16, TG73, TG53 |
| 2            | Light Yellow | TG63, TG64, TG65, TG66, TG67, TG80, TG50, TG8 |
| 3            | Light Brown  | TG103, TG91, TG112, TG122, TG125, TG18 |
| 4            | Cream        | TG7, TG107, TG108, TG130, TG131, TG110 |
| 5            | Orange       | TG69, TG70, TG71, TG21, TG54, TG22 |
| 6            | White        | TG31, TG117, TG118, TG95, TG126 |
| 7            | Grey         | TG104, TG105, TG106, TG87 |
| 8            | Ivory        | TG93, TG88, TG89, TG97 |
| 9            | Dark Yellow  | TG23, TG72, TG113 |
| 10           | Beige        | TG47, TG114, TG51 |
| 11           | Light Purple | TG4, TG6, TG5 |
| 12           | Light Red    | TG2, TG52, TG60 |
| 13           | Light Green  | TG14, TG15, TG46 |
| 14           | Pink         | TG20, TG123 |

Table 6. Assignment of actinomycete isolates to multimembered and single-membered colour-groups.

| Colour Group | Colony Colour | ID Actinomycete Isolates |
|--------------|--------------|--------------------------|
| 1            | Cream        | BG007, BG008, BG011, BG016, BG037, BG065(1), BG071, BG088, BG090, BG104, BG105, BG107, BG126, BG142 (1), BG169 |
| 2            | Dark Red     | BG004, BG042, BG064, BG066, BG075, BG082, BG089, BG115, BG152, BG153, BG160 |
| 3            | Beige        | BG019, BG034, BG072, BG110, BG112, BG132, BG182, BG187, BG189 |
| 4            | Pale Yellow  | BG010, BG020, BG036, BG085, BG094, BG143, BG155, BG165 |
| 5            | Light Brown  | BG002, BG015, BG035, BG057, BG109, BG127, BG149, BG161 |
| 6            | Brown        | BG032, BG087, BG101, BG118, BG123, BG163, BG171, BG180 |
| 7            | Yellow       | BG058, BG065(2), BG097, BG124, BG140, BG145, BG146, BG159 |
| 8            | Red          | BG009, BG051, BG120, BG128, BG133, BG150 |
| 9            | Orange       | BG061, BG086, BG092, BG154, BG174 |
| 10           | Dark Green   | BG060, BG125, BG158, BG177 |
| 11           | White        | BG014, BG083, BG114, BG137 |
| 12           | Light Brown  | BG038, BG095, BG136 |
| 13           | Dark Yellow  | BG043, BG080 |
| 14           | Orange       | BG151 |
| 15           | Dark Brown   | BG111 |

Based on the analyses of the dendrograms, there are thirteen bacterial phenetic multimembered groups recovered partially or completely by using both algorithms ($S_m$ and $S_j$). When the comparison was made between the colour and phenetic groups, there is a congruence of fourteen out of the sixteen phenetic groups. Regarding the analysis of the dendrogram for the actinomycete strains, there are nineteen phenetic groups that are found clustered together, either partially or completely, when both algorithms are used. About the coherence of the colour and phenetic multimembered groups, there is less agreement since only fourteen out of the twenty phenetic groups were found in both characterisations. In any case, these results show that there is great coherence between the colour
grouping and the results from the numerical taxonomy analyses from the phenotypic data, thus validating the colour determination and formation of preliminary colour groups of isolates when there is a large collection of microbes.

These results also show that the procedures used in this study allowed the cultivation of many bacteria and actinomycetes present in the soil samples contaminated with TPHs. Regarding the bacteria, this fact is relatively normal since other studies, which have used either culture dependant or molecular detection techniques, have demonstrated that there are numerous bacterial groups present in this type of soils [10]. However, for the actinomycetes, this is the opposite since studies about their presence in TPH contaminated soils is limited. In any case, this study opens the door to carry out more experiments to elucidate the role of the actinomycetes in oil-contaminated soils and to explore the true extent of their diversity in such habitats.

Figure 1. Dendrogram based on the analysis of phenotypic data of seventy-three bacterial strains. Clustering was achieved by the UPGMA (Unweighted Pair Group Method with Arithmetic Means) algorithm from a similarity matrix calculated using the single matching coefficient ($S_{SM}$). Phenetic groups were obtained at 90% similarity. The asterisks denote phenetic groups that were also recovered using the Jaccard’s coefficient ($S_{J}$).
Figure 2. Dendrogram based on the analysis of phenotypic data of ninety-three actinomycete strains. Clustering was achieved by the UPGMA (Unweighted Pair Group Method with Arithmetic Means) algorithm from a similarity matrix calculated using the single matching coefficient ($S_{SM}$). Phenetic groups were obtained at 90% similarity. The asterisks denote phenetic groups that were also recovered using the Jaccard’s coefficient ($S_J$).

4. Conclusions
A large diversity of bacteria and actinomycete strains was found in two contaminated soil with TPHs. This result is supported by the numerical taxonomy analyses of phenotypic data which divided the microbial culture collection in thirty-one and twenty-four bacterial and actinomycete phenetic groups. Besides the microbial diversity findings, it is also important to note that more than 90% of the isolates could use gasoline or oil as sole carbon and nitrogen source. This result could be of great importance later in the process of developing a bioproduct for bioremediation purposes.

5. References
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