Novel diesel-oil-degrading bacteria and fungi from the Ecuadorian Amazon rainforest

N. R. Maddela, M. Masabanda and M. Leiva-Mora

ABSTRACT

Isolating new diesel-oil-degrading microorganisms from crude-oil contaminated sites and evaluating their degradation capacities are vitally important in the remediation of oil-polluted environments and crude-oil exploitation. In this research, new hydrocarbon-degrading bacteria and fungi were isolated from the crude-oil contaminated soil of the oil-fields in the Amazon rainforest of north-east Ecuador by using a soil enrichment technique. Degradation analysis was tracked by gas chromatography and a flame ionization detector. Under laboratory conditions, maximum degradability of the total n-alkanes reached up to 77.34 and 62.62 removal ratios after 30 days of incubation for the evaporated diesel oil by fungi (isolate-1) and bacteria (isolate-1), respectively. The 16S/18S rDNA sequence analysis indicated that the microorganisms were most closely (99–100%) related to Bacillus cereus (isolate-1), Bacillus thuringiensis (isolate-2), Geomyces pannorum (isolate-1), and Geomyces sp. (isolate-2). Therefore, these strains enable the degradation of hydrocarbons as the sole carbon source, and these findings will benefit these strains in the remediation of oil-polluted environments and oil exploitation.

Key words | bacteria, biodegradation, diesel, fungi

INTRODUCTION

Ecuador is the fifth largest oil producer in South America, and it has one of the highest rates of biodiversity of all Amazonian countries. It pumps 500,000 barrels of crude oil per day. Concurrently, massive oil drilling caused serious ecological risks in the world’s most sensitive and biodiverse ecosystems. In the present study, we focused on the Lago Agrio oilfield in an oil-rich area, located in the Western Oriente Basin in the province of Sucumbíos, Ecuador. This field is known internationally for the serious ecological problems that oil development has created there, including water pollution, soil contamination, deforestation and cultural upheaval (Gesinde et al. 2008). This has occurred because oil is a major source of income for the country and since the 1970s it has been the engine of the economy. This leads to the outpouring of a huge amount of crude oil (30 b gallons of toxic waste) into the environment (Sebastian & Hurtig 2004). Although the oil-companies take precautions and use remediation procedures, impacts are still evident (James 2013).

Another major reason behind this research work is because of the biodiversity of Ecuador. It is the eighth most biodiverse country on the planet, and one of 17 mega diverse countries in the world, according to Conservation International, with the highest species diversity per unit area (Conservation International 2003). In addition to past experience, recently, Ecuador has signed permits for oil drilling in the Amazon’s Yasuni national park, which spans about 9,820 km², and is arguably the most biologically diverse spot on Earth. Nonetheless, logging and oil exploitation could threaten to destroy a great deal of this natural wealth (Butler 2012). However, until now, very few research activities have been carried out in Ecuador (Buccina et al. 2013), which are not sufficient to understand the relationship between indigenous microorganisms and petroleum hydrocarbons (PHs). Thus, the isolation and characterization of PH-degrading microorganisms is not only of interest, but also requisite.

For the past 40 years, bioremediation has been the go-to method for cleaning contaminated sites. The major microorganisms responsible for biodegradation of PHs have been found to be bacteria and fungi. There are approximately 70 genera of known oil-degrading microorganisms, including bacteria such as Achromobacter, Acinetobacter, Actinomyces, Bacillus, Burkholderia, Exiguobacterium, Klebsiella,
Microbacterium, Nocardia, Pseudomonas, Spirillum, Streptomyces and Vibrio, and fungi or yeast such as Allescheria, Aspergillus, Candida, Debaryomyces, Mucor, Penicillium, Saccharomyces and Trichoderma. A consortium of two or more microbial cultures has proven even better results in most studies (Franzmann et al. 2002). In addition, even if PH-degrading microorganisms were absent from polluted sites, bioremediation was still reported through the bioaugmentation process (Supaphol et al. 2006). Therefore, from the literature, it is clearly understood that the PH-degrading microorganisms are requisite for the cleaning of contaminated sites by any means of microbiological remediating processes. To our knowledge, however, there remains a lack of information on bioremediation of crude-oil contaminated soils, ecotoxicity and reclamation of bioremediated soil in Ecuador. Thus, in this review, we have focused on the Lago Agrio oilfield, which is the biggest oil field in this country, and thousands of tonnes of oily sludge are generated and piled up on the ground every year. For that reason, there is an urgent need to devise effective methods to treat polluted sites, as well as to protect the great biodiversity of the Amazon rainforest. Therefore, the present study was initiated with the aim of isolating potential diesel-oil utilizing bacteria and fungi from crude-oil contaminated sites in Ecuador for the purpose of bioremediation in the near future.

MATERIALS AND METHODS

Soil collection

Soil samples (400 g) from surface soil (0–15 cm depth) were collected from different regions in Lago Agrio (Sarayaku community; E 974576,881 N 10001527,083; Figure 1), which was contaminated (Test) with crude oil due to extensive drilling and transportation of oil products. Similarly, uncontaminated soil samples were also collected in the same area (Control). Samples were taken from three or four random locations per plot. Prior to testing, the soils were air dried, passed through a 2 mm sieve and stored at 4 °C.

Physico-chemical and microbiological properties of soil samples

The physico-chemical and microbiological properties of two selected soil samples were determined using standard procedures (Nagaraju et al. 2009) and are listed in Table 1. The total number of heterotrophic bacteria and fungi present in the contaminated and uncontaminated soils were determined (Table 1) by using serial dilution and the plate counting technique (Lorch et al. 1995). Nutrient agar medium (NA) and

Figure 1 | Sampling sites (courtesy of Petroamazonas).
Crude-oil-contaminated soil of 1 g was dissolved in 10 mL of sterile saline solution and mixed thoroughly. Then, 2.5 mL of supernatant was transferred into 50 mL of Luria-Bertani broth containing 1% diesel oil. It was incubated at 37°C for 48 hours in a shaker at 100 rpm. To obtain the cell pellet, the broth was centrifuged at 5,000 rpm for 10 minutes. The pellet was washed twice with phosphate buffer (pH 6.8, 0.1 M), then dissolved in a small volume of a Bushnell Hass (BH) broth medium. Thereafter, 0.1 mL of this suspension was used to inoculate a BH agar plate containing 0.1 mL of diesel oil. Finally, the plates were kept at 37°C for 1 week. Pure cultures of diesel-oil-degrading bacteria (Figure 2(a) and 2(b)) were isolated and preserved at −80°C using 25% glycerol. Similarly, diesel-oil-degrading fungi (Figure 2(c) and 2(d)) were isolated by the following method. Mineral salt medium

### Table 1 | Physico-chemical and microbiological properties of soil samples

<table>
<thead>
<tr>
<th>Character</th>
<th>Control</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.12 ± 0.07</td>
<td>7.34 ± 0.01</td>
</tr>
<tr>
<td>Electrical conductivity (μs/cm)</td>
<td>88.4 ± 5.0</td>
<td>178.9 ± 9.0</td>
</tr>
<tr>
<td>Phosphorus (mg/kg)</td>
<td>17 ± 0.9</td>
<td>48 ± 0.9</td>
</tr>
<tr>
<td>Potassium (mg/kg)</td>
<td>332.98 ± 9.0</td>
<td>457.48 ± 5.0</td>
</tr>
<tr>
<td>Magnesium (mg/kg)</td>
<td>758.96 ± 16.0</td>
<td>949.43 ± 20.0</td>
</tr>
<tr>
<td>Calcium (mg/kg)</td>
<td>1,349.93 ± 26.0</td>
<td>2,999.85 ± 39.0</td>
</tr>
<tr>
<td>Organic matter (%)</td>
<td>9.74 ± 0.1</td>
<td>13.21 ± 0.6</td>
</tr>
<tr>
<td>Total nitrogen (%)</td>
<td>0.21 ± 0.05</td>
<td>0.21 ± 0.06</td>
</tr>
<tr>
<td>Lead (mg/L)</td>
<td>2.36 ± 0.09</td>
<td>3.44 ± 0.03</td>
</tr>
<tr>
<td>Cadmium (mg/kg)</td>
<td>15.76 ± 0.9</td>
<td>0.38 ± 0.2</td>
</tr>
<tr>
<td>Chromium (mg/kg)</td>
<td>0.08 ± 0.01</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>100% Water-holding capacity (mL/g)</td>
<td>0.486 ± 0.05</td>
<td>0.416 ± 0.04</td>
</tr>
<tr>
<td>Bacteria (cfu g⁻¹)</td>
<td>(3.2 ± 0.1) × 10⁸</td>
<td>(7.2 ± 0.2) × 10⁷</td>
</tr>
<tr>
<td>Fungi (cfu g⁻¹)</td>
<td>(4.8 ± 0.2) × 10⁶</td>
<td>(8.0 ± 0.1) × 10⁴</td>
</tr>
</tbody>
</table>

Note: Each value represents the average of three replicates (n = 3).

**Figure 2** | Microscopic observation of microorganisms: (a) bacterial isolate-1, (b) bacterial isolate-2, (c) fungal isolate-1, and (d) fungal isolate-2.
(MSM) was used as a culture medium (pH 5.5), which had the following composition (in g L⁻¹ distilled water): K₂HPO₄·3H₂O, O, 3; KH₂PO₄, 3; NaCl, 0.5; NH₄Cl, 1; MgSO₄·7H₂O, 0.5; CaCl₂ (trace); FeCl₃ (trace); chloramphenicol, 0.3. A quantity of fresh oil-contaminated soil was added into 50 mL sterilized MSM containing 1% diesel oil in an Erlenmeyer flask. Flasks were shaken at 120 rpm at 25 °C to homogenize the medium for 2 weeks for microbial enrichment; the aliquot of 2 mL enriched cultures was inoculated into another 100 mL Erlenmeyer flask containing 50 mL fresh MSM with the same amount of oil for the second enrichment. Finally, mycelia were filtered through filter paper under sterile conditions and preserved at −80 °C by using 25% glycerol.

Identification of microorganisms

Diesel-oil-degrading microorganisms were identified through 16S/18S rDNA sequence analysis. Two isolates of bacteria were cultured for 3 days on NA. Genomic DNA was extracted as previously described (Frederick et al. 1998) and DNA concentration measured with a UV-Vis spectrophotometer (GENESYS™ 10S UV-Vis Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). Polymerase chain reaction (PCR) was performed using the primers 27F and 1,492R that identify the 16S rRNA region (bacteria and archaea). The quality of the PCR product was found by running it on agarose gel of 2%, and fungal genomic DNA was extracted (Cenis 1992), and quantified using NanoDrop to make the required dilutions. Then, PCR was performed using the same protocol, then internal transcribed spacer 1 (ITS) and ITS2 were used to identify the ITS regions of fungal isolates. The amplified product was separated and purified using 2% agarose (A9529 SIGMA). Finally, PCR products obtained from the genomic DNA of both bacteria and filamentous fungi were sent to CIBE, ESPOL, Guayaquil (Ecuador) for further sequence analysis.

Experimental design

Experiments were performed in 100 mL Erlenmeyer flasks, containing 20 mL of liquid minimal salts medium (see above) plus 1% of diesel oil as a sole carbon source. Mycelia plugs of selected fungi (three 5-mm discs) were cut from the outer edge of an actively growing culture on an inoculum plate and inoculated into an Erlenmeyer flask containing sterilized medium. Then, these flasks were incubated at 25 °C. Abiotic control experiments were performed by incubating oil in minimal salts basal (MSB) medium without inoculums. All assays were conducted in triplicate. Before incubation, a flask of each treatment was selected for immediate extraction. All remaining flasks were incubated for 30 days. After incubation, the culture broth was blended with ethyl acetate and acidified with 1 N HCl. The filtrate (liquid medium) and residue (fungal body) were separated by filtration, and the liquid medium and fungal bodies were extracted with ethyl acetate, respectively. Then, extracts were purified by silica gel column chromatography using dichloromethane (DCM). Finally, these extracts were concentrated and analyzed by gas chromatography and flame ionization detector (GC/FID; Clarus 680 GC, PerkinElmer, Waltham, MA, USA) for total petroleum hydrocarbons (TPHs).

To test the bacterial cultures, 1% diesel oil was added to 20 mL of MSB media (Stanier et al. 1966). Prepared samples were grown at room temperature with shaking at 220 rpm. The residual diesel-oil concentration was analyzed using GC/FID. Culture solutions were collected at two intervals (0- and 30-days) and mixed with 5 mL of DCM. The culture-DCM mixture was stirred and centrifuged at 1,540 g for 10 minutes. After the separation, the DCM layers were filtered through nylon 66 filters (0.45 μm; Whatman, Little Chalfont, UK). Analytes were assayed using gas chromatograph. Finally, the biodegradation of diesel oil by fungi and bacteria was expressed in terms of a removal ratio (RR) (Joo et al. 2008), calculated as follows:

\[
\text{RR} = \frac{(\text{Initial concentration} - \text{Residual concentration})}{\text{Initial concentration}} \times 100\%
\]

Statistical analysis

The data were analyzed for significant differences (p < 0.05) between treatments, and isolates using analyses of variances. Minitab® 17 statistical software (Minitab Inc., State College, PA, USA) was used for statistical analyses in this study, and the test for significance between means is implied at α = 0.05 unless stated otherwise.

RESULTS AND DISCUSSION

Physico-chemical and biological properties

Test soil has shown significant differences in all studied parameters over control soil (Table 1). Discharge of crude-oil waste increased the soil pH to neutral (pH 7.34) from acidic (pH 5.12). The same result was reported by Obire & Nwaubeta (2002) where the total mean pH for the control soil was pH 5.9, while those for gasoline, kerosene, and diesel-oil contaminated soils were pH 6.5, 6.31 and 6.6, respectively. Therefore, the pH of the PH-contaminated soils is usually higher than the pH of the control soil.
Similarly, twofold higher electrical conductivity was recorded in the test soil compared to the control. The quantity of phosphorus, potassium, magnesium, calcium, organic matter and lead were much higher in the test sample than in the control soil (Table 1). However, the contents of total nitrogen (0.21%) and chromium (0.08 mg kg\(^{-1}\)) were not altered between two soil samples.

The content of cadmium, water-holding capacity, and microbial populations had been adversely affected in test soil compared to the control. Nearly 100 times fewer heterotrophic bacteria and fungi were observed in the test soil than in the control sample (Table 1). This indicates toxic concentrations of pollutants (Petrovic et al. 2008). It is evidence that PHs are usually not a good source of carbon for most of the microorganisms in soil, and they are toxic to them (Jorgensen et al. 2000). However, it is not true in all cases. An increased number of viable microorganisms has been noticed upon the addition of PHs to the soil (Chaineau et al. 2008). Obire & Nwaubeta (2002) reported that the addition of PHs to soil increased the microbial population, and that the cumulative bacterial count in the treated soils was in the order of kerosene > gasoline > diesel > control soil. Song & Bartha (1990) and Al-Awadhi et al. (1996) found a strong correlation between microbial counts and hydrocarbons degradation. Thus, the interaction between microorganisms and hydrocarbons varies from region to region, and it depends mostly on their quality and quantity.

**Identification**

Phylogenetic analysis based on 16S rDNA sequences of two bacterial isolates showed high similarity (99–100%) with the genus *Bacillus*. For instance, isolate-1 and -2 were identified as *B. cereus* (Accession Number: EF582416.1), and *B. thuringiensis* (AN: CP010089.1), respectively. On the other hand, fungal isolates were closer to the genus *Geomyces*. Isolate-1 was identified as *G. pannorum* (AN: JF320819.1) with 99% similarity, whereas, isolate-2 was also close enough to *Geomyces* sp. Although several bacteria (Bassim & Shquirat 2008) and fungi (Romero et al. 2010) were reported previously with PH-degrading abilities, there is no information for PH-degrading microorganisms for Amazonian Ecuador in the literature. Undoubtedly, this information will be a useful reference for all future studies in this region.

**Biodegradation of diesel oil by fungi and bacteria**

Results of this short-term *in vitro* study revealed that the isolated fungi and bacterial strains had the capacity to degrade diesel oil (Figure 3 and Table 2). According to the GC/FID analysis, the rate of biodegradation of diesel oil by fungi was higher than by the bacteria. Under the given conditions, the RRs of diesel-oil for isolate-1 and -2 of fungi and bacteria were 77.34, 68.55, 62.62 and 49.71, respectively (Table 2). Thus, RRs were significantly (*p* < 0.05) higher for fungi compared to bacteria. In particular, isolate-1 was as effective as isolate-2, in each category of microorganism.

More often than not, diesel oils are thought to be good carbon sources for enriching alkane degraders, which might have potential as a bioremediation agent in a crude-oil-contaminated site (Kang et al. 2009). This is true because large fractions of crude oil consist of long-chain alkanes (Kang et al. 2009). More recently, Al-Nasrawi (2012) found that there were different weight losses with fungal strains after three weeks of incubation. *Aspergillus niger* demonstrated the highest weight loss (8.6%) with *Penicillium decumbens* (7.9%) and *Cochliobolus lunatus* (4.7%), whereas the lowest weight loss was demonstrated by *Fusarium solani*, strain 421502 (1.9%). In 2008, Gesinde’s team confirmed the ability of *A. niger* to biodegrade crude oil with 18% weight loss and *P. notatum* with 11.2% weight loss; this indicates that these isolates have the potential to utilize crude oil as a carbon source. Likewise, researchers compared bacterial growth despite the presence of different PHs as a sole carbon source (Yakimov et al. 2011). In fact, bacteria were the primary degraders of spilled oil in the environment (Brooijmans et al. 2009).

The other fact understood from the current research results is that the rate of degradation of PHs was a little slower in the initial incubation. This is particularly true for the case of bacterial isolate-2 (Figure 3(e)). Nevertheless, all four strains have proven their degradation capacities. According to the chromatograms (Figure 3), it is also known that while PHs were not completely mineralized, they were partially degraded. Indeed, partial degradation also helps in the reduction of toxicity of PHs in the soil, if the produced metabolites do not cause harm to the soil biota.

For instance, a chromatogram in Figure 3(a) shows typical *n*-alkane peaks from C10–C18. In contrast, chromatogram from residual petroleum extracted from MSM (Figure 3(b)) at the conclusion of the experimental incubation period were noticeably weathered, lacking components more volatile than *n*-C12, and exhibiting highly degraded *n*-alkanes; in addition to this, new peaks were observed, which correspond to the new metabolites. Therefore, in the present investigation, we found that isolated fungal strains could utilize petroleum components as a carbon source. Much-degraded *n*-alkanes have been observed in other chromatograms (Figure 3(c)–3(e)) too.
could be even better if an incubation period was prolonged. Even better results have been observed by many researchers under long-term field experiments. It is also well known that no one species of microorganism will completely degrade any particular oil (Colwell & Walker 1977). Of the various petroleum fractions, \( n \)-alkanes of intermediate length (C10–C20) are the preferred substrates and tend to be most readily degradable (Singer & Finnerty 1984), whereas shorter chain compounds are rather more toxic (Klug & Markovetz 1971). Therefore, hydrocarbons differ in their susceptibility to microbial attack. The susceptibility of hydrocarbons to microbial degradation can be generally ranked as follows: linear alkanes > branched alkanes > small aromatics > cyclic alkanes (Ulrici 2000). Some compounds, such as the high molecular weight polycyclic aromatic hydrocarbons (PAHs), may not be degraded at all (Atlas & Bragg 2009). In conclusion, although the microbial cultures of the present study did degrade diesel oil

![Figure 3](https://iwaponline.com/wst/article-pdf/71/10/1554/468354/wst071101554.pdf)

**Figure 3** GC fingerprinting of TPHs of diesel oil. (a) Only diesel oil, (b) G. pannorum, (c) Geomyces sp., (d) B. cereus, (e) B. thuringiensis.
We isolated bacteria and fungi (two strains of each) from crude-oil contaminated soil in the Ecuadorian Amazon rainforest; these strains used diesel oil as a sole carbon source under laboratory conditions. Cultures were significantly ($p < 0.05$) effective in degrading alkanes, although the RRs were different. In particular, fungal isolate-1 has shown the highest RR (77.34) for the TPHs of diesel oil. Since all strains degrade diesel oil, there exists a possibility for their use in the development of microbial technology for decontamination of crude-oil-contaminated sites in Ecuador. The 16S/18S rDNA sequence analysis revealed that the isolates were closer to genera Bacillus and Geomyces. However, future studies will be required to address the microbial response to the field level in an Amazon rainforest, and the efficacy of co-substrates to stimulate PH biodegradation; these will give clues for effective bioremediation of crude-oil-contaminated soil.

CONCLUSIONS

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