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Removal of petroleum hydrocarbons from crude oil in solid and slurry phase by mixed soil microorganisms isolated from Ecuadorian oil fields

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ABSTRACT

Soil collected from oil fields of the Ecuadorian Amazon rainforest contained microorganisms capable of removing total petroleum hydrocarbons (TPHs) from crude oil. Following 16/18S rDNA sequence analysis, soil microorganisms efficient in the removal of TPHs were identified as *Bacillus cereus*, *Bacillus thuringiensis*, *Geomyces pannorum*, and *Geomyces* sp. A mixed culture of the above two isolates of bacteria and two of fungi were tested for its ability to remove TPHs from crude oil in solid phase (SOP) or slurry phase (SLP) of soil. The capability of the mixed culture in removing TPHs after 30 d incubation was higher in SLP than in SOP. Results of ecotoxicity studies using *Artemia salina* corroborated with those of TPHs removal from crude oil in SOP and SLP by the mixed culture of the selected bacteria and fungi.

1. Introduction

Bulk oil production in Ecuador resulted in the extensive pollution of soil, rivers and streams, and groundwater, and the local indigenous and *campesino* communities suffered with cancer epidemics, birth defects, miscarriages, and other ailments (Butler, 2012). Local tribes and wild life are under threat due to the movement of country's drilling operations to the Yasuni National Park, one of the world's most ecologically complex and fragile places. However, in this region, knowledge on microbial remediation technologies for cleaning the sites contaminated with chemically-complex heterogenous molecules of petroleum hydrocarbons (PHs) is very limited. For instance, there are some evidences for bioremediation of non-PHs contaminated soils (Russell et al., 2011), microbial systematics of crude oil contaminated soils, and bioremediation through land farming and biopiles combined with bioaugmentation (Barragán et al., 2008). But, there is no information on the role of indigenous microorganisms from the Amazonian Ecuador in degradation of PHs, especially in bioslurries. Permanent removal of hazardous pollutants from the environment is always a difficult task though it is not impossible. In recent times, much attention has been paid on bioremediation of oil-contaminated sites using various microbial species and innovative methods (Megharaj et al., 2011; Nikolopoulou et al., 2013; Silva-Castro et al., 2015). More than 70 species of *Bacillus* were identified as PHs degraders (Romanowska et al., 2015); however, the role of fungal species such as *Geomyces* sp. in degrading PHs has not been fully established (Hughes and Bethan, 2005).

The efficiency of microorganisms in degrading PHs is generally investigated in soil microcosms following solid phase (SOP) bioremediation, but rarely by slurry phase (SLP) bioremediation (Nano et al., 2003). Water phase is added in SLP to enhance the physical mixing, whereas in SOP only nutrients are added and the soil bed is agitated mechanically at intervals (Megharaj et al., 2014). Nonetheless, SLP bioremediation is much faster than many other bioremediation techniques (Eziuzor and Okpokwasili, 2013; Saez et al., 2014), and is very useful in cases where the contaminants need to be broken down very quickly. Another advantage of SLP

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bioremediation is the fact that it can be a permanent solution for the problem of pollution. By considering the extent and consequences of crude oil pollution on environment in the Ecuador, an attempt has been made to isolate efficient indigenous bacteria and fungi that remove PHs from crude oil contaminated soil, and remediate crude oil-treated soil in both the systems of solid and slurry phase.

2. Methods and materials

2.1. Isolation and identification of PHs-degrading microorganisms

Bacteria and fungi that degrade PHs were isolated from the crude oil-contaminated soil collected at random in 5–6 locations to a depth of 0–15 cm at the Lago Agrio oil field, Ecuador. Mixed soil samples were shade-dried and sieved through a 2 mm mesh. A composite soil sample thus obtained was used to determine the texture (Alexander, 1977), electrical conductivity (EC), pH in soil:-water (1:1) extracts (Thomas, 1996), water-holding capacity (WHC) (Johnson and Ulrich 1960), organic carbon by Walkley and Black method (Nelson and Sommers, 1996), total nitrogen following MicroKjeldahl method (Jackson, 1973), and available phosphorus and potassium (Lu, 1999). Total number of heterotrophic bacteria and fungi present in the oil-contaminated and uncontaminated soil samples were determined following serial dilution and plate counting technique (Lorch et al., 1995). Nutrient agar and Czapek Dox agar media were used for the viable estimates of bacteria and fungi, respectively.

To isolate efficient PHs-degrading bacteria and filamentous fungi, the method described by Mukherjee and Das (2005) was adopted. Briefly, 1.0 g of crude oil-contaminated soil was mixed with 100 ml of modified M9 medium (6.4 g $Na_2HPO_4.7H_2O$; 1.5 g KH_2PO_4; 0.25 g NaCl; 0.5 g NH_4Cl; 0.2 ml of 1 M $MgSO_4$; 10 μL of 1 M $CaCl_2$; final volume adjusted to 100 ml with distilled water) containing 1% (v/v) diesel in a 250 ml Erlenmeyer flask, incubated at 37 °C in an orbital shaker (200 rpm). We used diesel because it is a well-known soil pollutant with less volatility and abundant amounts of readily degradable hydrocarbons (Van De Steene et al., 2007; Kang et al., 2009; Maddela et al., 2015). An aliquot of 0.1 ml of suspension was spread onto nutrient agar medium (N9405 Fluka), and incubated at 37 °C for 4 d. Morphologically, six predominant bacteria were selected, and axenic cultures were developed by repeated subculturing on agar slopes. These isolates were incubated individually at 37 °C in minimal salts medium supplemented with 1% diesel to test for their capabilities in degrading PHs. In order to isolate PHs-degrading filamentous fungi, 1.0 g of soil sample was homogeneously mixed with 0.1 ml of Tween-80 (Sigma Aldrich), a loopful of this sample was sprinkled on Sabouraud dextrose agar (SDA) plates (Fluka) and the plates were incubated at 28 °C for 5 d. Three distinct cultures were subcultured onto fresh SDA plates to obtain pure cultures, and screened for their PHs-degrading abilities following modified method proposed by Hanson et al. (1993). Finally, two isolates each of bacteria and fungi (Fig. 1a), efficient in degrading PHs in diesel were preserved at −80 °C using 25% (v/v) glycerol.

Genomic DNA from the bacterial isolates was extracted as described by Frederick et al. (1998) and PCR was performed using the primers 27F and 1492R for amplifying the 16S rRNA region. Fungal genomic DNA was extracted from the two isolates by the method of Cenis (1992) and PCR product was obtained using internal transcribed spacers 1 (ITS1) and 2 (ITS2) to identify the ITS regions of fungal isolates. PCR products obtained from the genomic DNA of both bacteria and filamentous fungi were sent to CIBE, ESPOL, Guayaquil (Ecuador) for sequence analysis and identification.

2.2. Removal of TPHs from crude oil by mixed microbial cultures

Laboratory-scale remediation of soil, treated with crude oil, by a mixed culture of two selected bacterial isolates and two fungal isolates was performed as detailed below. About 500 g of soil, sterilized by autoclaving, was mixed with 10% crude oil (API gravity 21.4, Petroamazonas EP) as has been used earlier by Mukherjee and Bordoloi (2011), and layered (5 cm depth with 50% WHC) in rectangular trays (30 × 40 × 10 cm) (Fig. 1b). Crude oil-treated soil samples also received 50 ml of Tween-80 (1.0 g L^{-1}, w/v), and inorganic sources of nitrogen (NH_4NO_3) and phosphorus (KH_2PO_4/Na_2HPO_4) to provide an initial C/N/P ratio of approximately 100:10:1 (Vezquez et al., 2009). After leaving the treated soil samples for 72 h at ambient temperature and 75–85% humidity, 25 ml of microbial inoculum in mixture was added to 500 g of soil and thoroughly mixed. Inoculum was prepared by mixing equal volumes of each of the four isolates with an initial bacterial suspension of 1.0 OD_{600} and fungal spore suspension containing 1×10^4 spores ml^{-1}. Crude oil-treated soil samples without the addition of inoculum served as controls. The treated soil samples in triplicates contained in the trays were thoroughly tilled at a regular interval of 5 d, and mineral salts medium was sprayed for maintaining moisture (50%) and nutrient levels. Soil samples in each tray also received 25 ml of glucose solution (250 mg L^{-1}) after 10 and 20 days since supplemental glucose serves as a co-carbon source and enhances the rate of degradation of polycyclic aromatic hydrocarbons (PAHs) by microorganisms during bioremediation (Das and Mukherjee, 2007). In the experiment related to SLP (Fig. 1c), each Erlenmeyer flask (250 ml) received 100 ml of 10% sterilized soil suspension along with all the amendments as in SOP setup. The soil suspensions were inoculated with a mixed culture (5 ml) of all the four microorganisms per 100 ml of soil slurry. Crude oil-treated samples of SLP that received no inoculum served as controls. All the flasks were shaken (120 rpm) in an orbital shaker at room temperature for 30 d. Triplicates of treated soil samples both from SOP and SLP were used to extract and analyze petroleum hydrocarbons at the start of the experiment (0 day) and after 30 days of incubation at room temperature by the methods described by Mukherjee and Bordoloi (2011) and Joo et al. (2008), respectively. Viable estimates of PHs-degrading bacteria and fungi present at the end of 30 days were also determined following the methods described by Margensin et al. (2003).

2.3. Ecotoxicity tests

Toxicity of PHs present in the soil samples at the start (0 day) and after bioremediation (30 d) was tested using brine shrimp, *A. salina*, for the bioassay (Nunes et al., 2006; Silva et al., 2014). Initially, residual toxicity was determined with the use of original undiluted soil samples by monitoring the per cent viability of animals for specific time intervals of 1 and 24 h. Then, 24h-LD_{50} was determined with different dilutions (1:1 to 1:10) of each soil sample prepared in 2% NaCl (pH 8.5). Ten milliliters of each soil dilution, filtered through Whatman No. 1 paper, were taken into Petri dishes, and ten actively growing nauplii (Fig. 1d) were added to each dish. After 24 h of incubation at 28 °C, LD_{50} concentrations were calculated by using dose–response curves (Cavalcante et al., 2013).

2.4. Extraction and analysis of TPHs

To determine TPHs at 0 and 30 d in soil samples by gas chromatography (GC), a wet sample (10 g dry equivalent weight) was mixed with anhydrous sodium sulfate (5 g) in an Erlenmeyer flask. After adding analytical grade hexane (100 ml), the flask was