Effect of process parameters on the bioremediation of diesel contaminated soil by mixed microbial consortia

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A B S T R A C T
This study focused on investigating the key parameters that influence carbon dioxide (CO2) production during the bioremediation of diesel contaminated soil. The effects of diesel concentration, moisture content and biomass dose were investigated in batch experiments, for 20 days, to ascertain the CO2 production and the amount of diesel mineralized. A regression model based on full factorial design of experiments was developed to predict the CO2 production. Based on the F and p values from Analysis of Variance (ANOVA) results, the main effects of process parameters affected diesel bioremediation strongly than the 2-way and 3-way interaction effects. The highest total petroleum hydrocarbon (TPH) mineralized and the maximum CO2 productions were ~3000 mg/kg-soil and ~10,000 mg/kg-soil, respectively, at a diesel concentration of 10,000 mg/kg-soil, 20% moisture content and a biomass dose of 275 mg/kg-soil.

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1. Introduction

Bioremediation of diesel contaminated soil by the use of microorganisms is an emerging environmentally friendly and cost effective technology. Diesel is a well known environmental pollutant that can easily contaminate soil and groundwater through their intrusion by various routes such as leakage from underground storage tanks, accidental spills, drilling sites, improper waste disposal practices, and leaching landfills (Roy et al., 2014). Hydrocarbon components released into the soil can be remediated by biodegradation processes (Chemlal et al., 2013). Biodegradation is defined as a biologically catalyzed oxidation or reduction reaction involving complex chemical compounds, based on either growth or co-metabolism. In the case of growth, organic pollutants are used as the sole source of carbon and energy which results in their complete degradation (mineralization). On the other hand, co-metabolism is defined as the metabolism of an organic compound in the presence of a growth substrate which is used as the primary carbon and energy source (Das and Chandran, 2011). Biodegradation of organic pollutants is governed by the activity of microorganisms such as bacteria, fungi, yeast and microalgae. These microorganisms co-exist with effective cooperation from the soil. However, the lack of microbial consortia strength (concentration) results in poor treatability of the soil contaminated with diesel (Maletic et al., 2011). Therefore, the inoculation of diesel contaminated soil with microbial consortia having high metabolic activity is a prerequisite to achieve effective bioremediation (Bento et al., 2005; Bundy et al., 2004; Rocha et al., 2000; Zanaroli et al., 2010).

Bioremediation of diesel contaminated soils can be achieved under in-situ or ex-situ conditions (Silva et al., 2015). In-situ bioremediation has proven to be cost-effective and often considered to be the safest method with limited disruption to field operations (Suja et al., 2014). Factors such as the availability of microorganisms with appropriate enzymatic and physiological capacity, favorable environmental and nutritional conditions for microbial growth and metabolism, the composition and concentration of the pollutants affects the efficiency of bioremediation processes. However, the bioavailability of active microbial consortia plays a major role to enhance diesel degradation rates. The lack of sufficient microbial population in diesel contaminated soil results...
in low biodegradation potential and slow biodegradation rates (Thompson et al., 2005).

An overview of literature on this topic indicated that biostimulation and/or bioaugmentation techniques enhance the efficiency of bioremediation of diesel contaminated soils. Biostimulation is the process in which phosphorus and nitrogen are introduced into the soil to initiate the growth of microorganisms in order to achieve faster biodegradation rates of diesel. On the other hand, bioaugmentation is the process in which potential microorganisms are inoculated in diesel contaminated soil to facilitate the biodegradation processes (Cerqueira et al., 2014; Silva-Castro et al., 2013; Sprocati et al., 2012). Besides, the addition of surfactant, hydrogen peroxide and other organic wastes such as wheat straw has shown to improve the bioremediation efficiency of diesel contaminated soils (Soleimani et al., 2013). In addition to biostimulation and bioaugmentation, chemical oxidation and electrokinetic oxidation techniques have also been suggested for the treatment of diesel contaminated soil (Falciglia et al., 2011).

Carbon dioxide (CO₂) production and their generation rate during bioremediation have been used as an index of microbial activity (Balba et al., 1998; Namkoong et al., 2002). It is a primary greenhouse gas emitted through the human activities and natural processes. An average global CO₂ flux from contaminated soil is about 68 ± 4 Pg-C/yr, whereas fossil fuel burning adds only about 5 Pg-C/yr. Thus, even a small change in the soil respiration flux may affect the annual loading of atmospheric CO₂ (Raich and Schlesinger, 1992). Soil organic matter contains approximately 1600 Pg carbon reservoir which is more than twice that of the atmospheric CO₂-C pool. Solomon et al. (2009) showed that the atmospheric CO₂ emission is likely to increase from current levels of 380 ppm (v/v) to peak values of 450–600 ppm (v/v) over the coming century and an irreversible sea level rise of at least 0.4–1.00 m is expected. Therefore, quantification of CO₂ gas that is emitted during bioremediation of diesel contaminated soil is of greatest concern from the sustainable bioremediation viewpoint.

In this study, the main and interaction effects of process parameters on diesel mineralization (Dm, %) and subsequent CO₂ production (μmol) in bioaugmented soil using experiments designed by full factorial design was performed by considering the diesel concentration, moisture content and biomass dose as the main parameters.

2. Materials and methods

2.1. Soil sample

Soil samples were collected from the University of UlSan (South Korea) garden at a depth of 8–10 cm and transported by a sterile plastic bag to the laboratory, and it was air dried and sieved using 1.18–425 μm sieve size in order to obtain homogenized soil samples prior to use.

2.2. Diesel

Diesel (density = 0.801 kg/L) sample was purchased from a local gasoline station and stored in the refrigerator (at 4 °C), under dark condition.

2.3. Microorganism

The mixed microbial consortium previously acclimatized to petroleum hydrocarbon was obtained from Ecophile, South Korea. It contained a consortia of Pseudomonas sp., Yarraia sp., Acinetobacter sp., Corynebacterium sp., and Sphingomonas sp. These cultures were concentrated through centrifugation (Eppendorf centrifuge 5804 R). The consortium had a total biomass concentration of 2.041 g/L.

2.4. Experimental design

A full factorial experimental design was formulated with three factors and two levels (2³) in order to investigate the effect of diesel concentrations (X₁), moisture content (X₂) and biomass dose (X₃) on diesel mineralization and CO₂ production. Each factor was investigated at two levels (low and high) and it was assumed that the response is approximately linear over the range of the factor levels considered. The statistical model for a 2³ design would include k main effects, k two factor interactions, and (k/3) three factor interactions. The ranges of the independent variables and the levels investigated using factorial design are shown in Tables 1 and 2, respectively. Considering the biomass dose applied for the different experimental runs, the diesel to microorganism (F/M) ratio was varied from 10 to 300. All statistical calculations (F - Fischer’s variance ratio, p - probability value, DF- degrees of freedom, Seq SS - sequential sum of squares and Adj MS - adjusted mean of squares) and analysis were done using the software MINITAB (Product version: Minitab 17.2.1, PA, USA).

2.5. Batch biodegradation studies

Biodegradation experiments were conducted in batch incubations by varying the diesel concentration, moisture content, and biomass dose from low and high levels, in 160 ml glass serum bottles (Wheaton, New Jersey, USA) (Table 2). 25 g soil was spiked with the desired concentrations of diesel and mixed with concentrated microorganism consortium. Known amounts of surfactant (Twee 80, 0.2% w/w) and water was added to the soil. Twee 80 was added in order to increase the bioavailability of the hydrocarbons to the microorganisms. Nitrogen and phosphorus were also added to the soil at a ratio of Oil: N: P of 100: 1: 0.2. The soil was vortex mixed to obtain a homogenized mixture. The serum bottles were closed air tight using rubber septa and aluminum crimps and incubated at 25 °C. Batch vials were purged with CO₂ free air (1.2 L/ min) by passing air through a KOH packed column followed by 4 M NaOH trap. A humidifier was used to prevent aspirated alkaline solution and provide 100% humidified air to the vials at room temperature (Namkoong et al., 2002).

2.6. Control experiments

To determine the activity of natural indigenous microorganisms on the bioremediation process, three sets of experiments with initial diesel concentrations of 5000 mg/kg-soil, 10,000 mg/kg-soil and 15,000 mg/kg-soil, under field moist conditions were investigated. To understand the soil background, respiration experiments having sample moisture contents ranging from 10 to 30% were incubated together with the batch bioremediation experiments. In order to assess abiotic diesel losses through physical processes, experiments were also conducted using oven dried soil.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Experimental parameters and levels investigated for the biodegradation of diesel.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Process parameters</td>
<td>Low level</td>
</tr>
<tr>
<td>Diesel concentration (mg/kg-soil), X₁</td>
<td>5000</td>
</tr>
<tr>
<td>Moisture content (%), X₂</td>
<td>10</td>
</tr>
<tr>
<td>Biomass dose (mg/kg-soil), X₃</td>
<td>50</td>
</tr>
</tbody>
</table>

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As a result of the elevated oven temperature was kept constant (320 °C) for 60 min. The gas chromatography was operated at oven temperature of 45 °C, held constant for 5 min, ramped to 100 °C at the rate of 5 °C/min and then up to 320 °C at a rate of 10 °C/min. Finally, the elevated oven temperature was kept constant (320 °C) for 40 min. The gas chromatography was performed by analyzing the standard gas mixture sample. The percentage of the individual peak area was measured to estimate the CO2 concentrations.

2.7.3. Diesel analysis

About 20 g of soil was sampled and mixed well with appropriate amount of Na2SO4 to absorb the water from soil matrix. Dichloromethane (100 ml) was used to extract diesel from the soil by ultrasonic sonication for about 6 min. The extractants were then filtered (110 mm filter paper) and concentrated to 20 ml via evaporation. 2 μl of the extracted sample was then injected into Gas chromatography DS6200 (Donam Instruments Inc, Korea) equipped with a capillary column (HP-5MS, crosslinked 5% PH ME Siloxane, 30 m × 0.250 mm × 0.5 μm) and a flame ionization detector (FID). The gas chromatography was operated at oven temperature of 45 °C, held constant for 5 min, ramped to 100 °C at the rate of 5 °C/min and then up to 320 °C at a rate of 10 °C/min. Finally, the elevated oven temperature was kept constant (320 °C) for 40 min. The gas chromatography was performed by analyzing the standard gas mixture sample. The percentage of the individual peak area was measured to estimate the CO2 concentrations.

Table 3

<table>
<thead>
<tr>
<th>Properties</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color</td>
<td>Dark brown</td>
</tr>
<tr>
<td>Grain size range (mm)</td>
<td>0.4–1.18</td>
</tr>
<tr>
<td>Water content (%)</td>
<td>12.8 ± 0.1</td>
</tr>
<tr>
<td>Field capacity (%)</td>
<td>33.0 ± 0.3</td>
</tr>
<tr>
<td>pH</td>
<td>6.9 ± 0.1</td>
</tr>
<tr>
<td>Organic carbon (%)</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>Total nitrogen (%)</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>Phosphorus (%)</td>
<td>8.3 × 10⁻⁴</td>
</tr>
<tr>
<td>C:N:P</td>
<td>100:6.8:0.04</td>
</tr>
<tr>
<td>Cation exchange capacity (meq/100 g)</td>
<td>5.1</td>
</tr>
</tbody>
</table>

Note.

a Field moist soil.
b Dry weight.

2.7.4. Mineralization of diesel during bioremediation of soil

Mineralization and CO2 production during bioremediation of diesel contaminated soil can be achieved by bioaugmentation process. Mineralization of diesel was measured as TPH and calculated using Eq. (2).

\[
\text{Mineralization of diesel (Dm)} = \frac{Df - Di}{Df} \times 100 \tag{2}
\]

where, \(D_m\) = Mineralization of diesel (%), \(D_i\) = Initial concentration of diesel (mg/kg-soil), and \(D_f\) = Final concentration of diesel (mg/kg-soil). Diesel concentration in the soil (mg/kg-soil) was measured as TPH content (mg/kg-soil).

3. Results and discussion

Bioremediation of diesel contaminated sites involves the use of biocatalysts to reduce high levels of diesel to levels that are innocuous and as a result, it minimizes the subsequent damages caused to the environment. It is a common knowledge that microbes require nutrients, carbon and energy sources to thrive in harsh polluted environments and specific microorganisms (both pure and mixed cultures) used for the bioremediation of diesel contaminated soils are not an exception. However, the biodegradation rates of these microbes depend on the hydrocarbon physicochemistry and environmental conditions such as temperature, pH, moisture content (Militon et al., 2010), bioavailability of the pollutant (Fernández et al., 2016), their contamination levels and the presence of additional nutrients (Franco et al., 2014). In addition...
to this, biotic factors such as competition between indigenous and exogenous microorganisms for limited carbon sources, as well as antagonistic interactions and predation by protozoa and bacteriophage determines the final outcome of the bioremediation process (Mrozik and Piotrowska-Seget, 2010).

3.1. Physicochemical characteristics of soil

The results from soil sample analysis showed that the soil had a characteristic dark brown color which is probably due to the decomposed leaf litter. The pH of the soil was 6.9 ± 0.1. It has been reported that such soil is ideal for hydrocarbon degrading microorganism to be adapted in the bioaugmentation process (Margesin and Schinner, 2005). The C: N: P ratio of soil was found to be 100: 6.8: 0.04, which indicates the lack of phosphorous sources (Table 3). The CEC was found to be lower (5.1 meq/100 g) than the ideal (10–30 meq/100 g) for agricultural soil (Miller and Miller, 1987).

3.2. Mineralization of diesel during bioremediation of soil

Table 4 shows the extent of diesel mineralization and CO2 production during the bioremediation of diesel contaminated soil by bioaugmentation. Mineralization of diesel was found to be highest (~67%) in experiments 9 and 10 (centre point) and lowest in experiment 3 (36.5%). The CO2 production was in the range of 2662–10,084 mg/kg-soil which corroborates with the results of Maletić et al. (2011). The favorable diesel concentration for the bioremediation of diesel-contaminated soil was approximately 10,000 mg/kg-soil. However, a slightly adverse effect on CO2 production was observed at a concentration of about 20,000 mg/kg-soil and toxic effect on microorganism was observed at diesel concentration of 35,000 mg/kg-soil. Lapinskiene et al. (2006) also reported that the toxicity of diesel on microorganism at diesel concentration of 35,000 mg/kg-soil. Lapinskiene et al. (2006) also reported that the toxicity of diesel on microorganism at diesel concentration of 35,000 mg/kg-soil, whereas, a loss of 15% diesel was observed at a diesel concentration of 10,000 mg/kg-soil (Eriksson et al., 2001). Chen et al. (2016) reported the simultaneous adsorption and biodegradation of diesel oil using modified bamboo charcoal (MBC) immobilized with Acinetobacter venetianus and reported a diesel mineralization efficiency of 94%. In that study, the reaction kinetics followed pseudo second order indicating the fact that diesel oil was first adsorbed onto bamboo charcoal and then degraded by the immobilized cells.

In another report, higher diesel concentrations did not significantly influence the diesel removal rate (Horel and Schiewer, 2009). To investigate the effect of diesel concentration, the authors spiked two soil samples with 0.2% and 0.4% (2 and 4 g of diesel/kg of soil) of diesel. According to the authors, although doubling the contamination levels resulted in doubling the cumulative mineralization of diesel in soil, their rate constants was nearly identical, i.e., 0.0041/d and 0.0043/d, for the high and low contaminated samples, respectively.

3.3. CO2 production during bioremediation

The bioremediation of soil with higher concentrations of diesel, i.e., by maintaining other parameters at constant values, usually resulted in higher CO2 production (Fig 3a). When the diesel concentration was increased from 5000 mg/kg-soil (experiment 3) to 15,000 mg/kg-soil (experiment 5), the CO2 production was increased from 10% (experiment 2) to 30% (experiment 4) by maintaining other parameters constant (diesel concentration: 5000 mg/kg-soil; biomass dose: 500 mg/kg-soil). It has been reported that increased moisture causes inhibition of microbial activity through impairment of gas diffusion in soil. This could be the reason for the decreased mineralization in the soil (Tibbett et al., 2011). Furthermore, mineralization of diesel increased by about 29% in experiment 6 (Biomass dose: 500 mg/kg-soil) as compared to that of experiment 5 (Biomass dose: 50 mg/kg-soil), at constant diesel concentration (15,000 mg/kg-soil) and moisture content (10%) (Fig 1b). This could be explained by the fact that higher the microbial dose, higher is the adaptability and assimilation capacity of microbial population to the newly introduced soil, which in turn results in higher mineralization efficiency (Trindade et al., 2002). Only about 16% of the diesel was mineralized in the natural soil. On the other hand, beneficially, mineralization of diesel by the bioaugmented soil was found to be 51% higher than the natural soil (Fig 2). Physical sorption of diesel (abiotic loss) in the oven dried soil was found to be 20.5%, 16.0% and 9.7%, respectively, at diesel concentrations of 5000 mg/kg-soil, 10,000 mg/kg-soil and 15,000 mg/kg-soil, respectively. The loss of TPH (mg/kg-soil) was estimated to be 468, 719 and 639 at diesel concentrations of 5000, 10,000 and 15,000 mg/kg-soil, respectively, which clearly indicates that the soil had the potential to sorb a certain amount of diesel, especially the clay and humus compounds present in the soil. This indicates the homogeneity of the sampled soil analyzed during the bioremediation process. Adsorption and desorption efficiency of diesel is usually affected by the type of soil texture (Falciglia et al., 2011). About 16–23% loss of diesel was reported during the bioremediation of soil at a concentration of 5000 mg/kg-soil (Margesin and Schinner, 1997; Hur and Park, 2003), whereas, a loss of 15% diesel was observed at a diesel concentration of 10,000 mg/kg-soil (Eriksson et al., 2001), Chen et al. (2016) reported the simultaneous adsorption and biodegradation of diesel oil using modified bamboo charcoal (MBC) immobilized with Acinetobacter venetianus and reported a diesel mineralization efficiency of 94%. In that study, the reaction kinetics followed pseudo second order indicating the fact that diesel oil was first adsorbed onto bamboo charcoal and then degraded by the immobilized cells.

### Table 4

<table>
<thead>
<tr>
<th>Expt run no</th>
<th>DC (mg/kg-soil) (X1)</th>
<th>MC (%) (X2)</th>
<th>BD (mg/kg-soil) (X3)</th>
<th>CO2 (mg/kg-soil)</th>
<th>TPHm (mg/kg-soil)</th>
<th>CO2/ TPHm (%)</th>
<th>Dm (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5000</td>
<td>10</td>
<td>50</td>
<td>3574</td>
<td>961</td>
<td>3.7</td>
<td>42.1</td>
</tr>
<tr>
<td>2</td>
<td>5000</td>
<td>10</td>
<td>500</td>
<td>5298</td>
<td>1208</td>
<td>4.4</td>
<td>53.0</td>
</tr>
<tr>
<td>3</td>
<td>5000</td>
<td>30</td>
<td>50</td>
<td>2662</td>
<td>832</td>
<td>3.2</td>
<td>36.5</td>
</tr>
<tr>
<td>4</td>
<td>5000</td>
<td>30</td>
<td>500</td>
<td>3184</td>
<td>917</td>
<td>3.5</td>
<td>40.2</td>
</tr>
<tr>
<td>5</td>
<td>15,000</td>
<td>10</td>
<td>50</td>
<td>3760</td>
<td>2666</td>
<td>1.4</td>
<td>40.2</td>
</tr>
<tr>
<td>6</td>
<td>15,000</td>
<td>10</td>
<td>500</td>
<td>5261</td>
<td>3456</td>
<td>1.5</td>
<td>52.3</td>
</tr>
<tr>
<td>7</td>
<td>15,000</td>
<td>30</td>
<td>50</td>
<td>3066</td>
<td>3435</td>
<td>0.9</td>
<td>52.0</td>
</tr>
<tr>
<td>8</td>
<td>15,000</td>
<td>30</td>
<td>500</td>
<td>3860</td>
<td>3550</td>
<td>1.1</td>
<td>53.7</td>
</tr>
<tr>
<td>9</td>
<td>10,000</td>
<td>20</td>
<td>275</td>
<td>10,084</td>
<td>3016</td>
<td>3.3</td>
<td>67.1</td>
</tr>
<tr>
<td>10</td>
<td>10,000</td>
<td>20</td>
<td>275</td>
<td>10,067</td>
<td>3043</td>
<td>3.3</td>
<td>67.7</td>
</tr>
</tbody>
</table>

Note.
DC: Diesel concentration; MC: Moisture content; BD: Biomass dose; CO2: Carbon dioxide production, TPHm: Total petroleum hydrocarbon mineralized; Dm: Diesel mineralized.
15,000 mg/kg-soil (experiment 7), about 15% increase in CO2 production was observed, at identical moisture content (30%) and biomass dose (50 mg/kg-soil) in both the cases. It was presumed that the increased diesel concentration served as a readily available organic source for the microorganism, and this organic source was mineralized to CO2 through biochemical activity. Moreover, about 25% decrease in CO2 production was observed when the moisture content was decreased from 30% to 10% (Fig 3b). Higher moisture content supports the agglomeration of loamy and sandy soil, thus reducing the gas diffusion through pores filled with water thereby inhibiting the microbial activity (Cho et al., 2000a; Freijer et al., 1996; Tibbett et al., 2011). A moisture content of 30% showed a

Table 5
Summary of the literature findings comparing CO2 production and diesel degradation during bioremediation of hydrocarbon contaminated soils.

<table>
<thead>
<tr>
<th>No</th>
<th>Type of hydrocarbon</th>
<th>Time (d)</th>
<th>Soil wt (g)</th>
<th>Concentration of contaminant (mg/kg-soil)</th>
<th>Moisture content (%)</th>
<th>Type of microorganism</th>
<th>CO2 production (mg/kg-soil)</th>
<th>Hydrocarbon mineralization (mg/kg-soil)</th>
<th>Hydrocarbon mineralization (%)</th>
<th>CO2/HCm References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Diesel</td>
<td>42</td>
<td>200</td>
<td>4000 (HC)</td>
<td>NA</td>
<td>Indigenous soil microorganism</td>
<td>5000</td>
<td>1600</td>
<td>40</td>
<td>3.125</td>
</tr>
<tr>
<td>2</td>
<td>Diesel</td>
<td>55</td>
<td>50</td>
<td>5561 (TPH)</td>
<td>16.6</td>
<td>Consortium (Ochrobacterium anthrophi, Stenotrophomonas maltophilia and Bacillus cereus)</td>
<td>6160</td>
<td>1100</td>
<td>20</td>
<td>5.6</td>
</tr>
<tr>
<td>3</td>
<td>Oil and Grease</td>
<td>70</td>
<td>1500</td>
<td>141,000 (O&amp;G)</td>
<td>20</td>
<td>Indigenous soil microorganism</td>
<td>6249</td>
<td>29,000</td>
<td>75</td>
<td>0.215</td>
</tr>
<tr>
<td>4</td>
<td>Crude oil</td>
<td>120</td>
<td>1875</td>
<td>170,000 (TPH)</td>
<td>23</td>
<td>Consortium (Pseudomonas putida, Bacillus subtilis)</td>
<td>119,466</td>
<td>78,400</td>
<td>74</td>
<td>1.52</td>
</tr>
<tr>
<td>5</td>
<td>Heating diesel fuel</td>
<td>28</td>
<td>1000</td>
<td>2000 (HC)</td>
<td>6</td>
<td>Diesel degrading cultures directly added as soil</td>
<td>1250</td>
<td>360</td>
<td>18</td>
<td>3.47</td>
</tr>
<tr>
<td>6</td>
<td>Oil residue</td>
<td>133</td>
<td>50</td>
<td>34,000 (O)</td>
<td>70 (FC)</td>
<td>7 Strains of Trichodaroma pseudokoningi, Eurotium amstelodami, Aspergillus versicolor, Aspergillus terreus, Cylindrocarpon didymium and 4 Strains of bacteria, Ochrobacterium anthrophi, Stenotrophomonas maltophilia, Bacillus cereus</td>
<td>5400</td>
<td>9390</td>
<td>28</td>
<td>0.575</td>
</tr>
<tr>
<td>7</td>
<td>Diesel</td>
<td>100</td>
<td>1000</td>
<td>10,000 (HC)</td>
<td>60 (FC)</td>
<td>Addition of compost</td>
<td>4250</td>
<td>8388</td>
<td>84</td>
<td>0.506</td>
</tr>
<tr>
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<td>Crude oil</td>
<td>16</td>
<td>20</td>
<td>100,000 (HC)</td>
<td>60 (FC)</td>
<td>Composting by poultry manure</td>
<td>1200</td>
<td>4608</td>
<td>7.72</td>
<td>0.26</td>
</tr>
<tr>
<td>9</td>
<td>Crude oil</td>
<td>45</td>
<td>300</td>
<td>9700 (TPH)</td>
<td>73 (FC)</td>
<td>Indigenous bacteria and biostimulation</td>
<td>4000</td>
<td>4365</td>
<td>45</td>
<td>0.916</td>
</tr>
<tr>
<td>10</td>
<td>Light crude oil</td>
<td>35</td>
<td>50</td>
<td>30,000 (HC)</td>
<td>66 (FC)</td>
<td>Corynebacterium, sphigomonosus and Yarrowia spp.</td>
<td>24,000</td>
<td>7800</td>
<td>26</td>
<td>3.07</td>
</tr>
<tr>
<td>11</td>
<td>Diesel</td>
<td>50</td>
<td>50</td>
<td>22,000 (HC)</td>
<td>50 (FC)</td>
<td>Commercial bacterial inoculums</td>
<td>22,000</td>
<td>10,920</td>
<td>54</td>
<td>2.014</td>
</tr>
<tr>
<td>12</td>
<td>Diesel</td>
<td>57</td>
<td>50</td>
<td>22,607 (HC)</td>
<td>21.1</td>
<td>Consortium of Staphylococcus hominis and Kocuria palustaris</td>
<td>6160</td>
<td>3987</td>
<td>23</td>
<td>1.54</td>
</tr>
</tbody>
</table>

Note. HC: Hydrocarbon; HCm: Hydrocarbon mineralized; TPH: Total petroleum hydrocarbon; O&G: Oil and grease; O: Oil; NA: Not applicable; FC: Field capacity.

Fig. 1. Comparison of mineralization of diesel at: (a) different moisture contents (diesel concentration - 5000 mg/kg-soil and biomass dose - 500 mg/kg-soil), and (b) different biomass dose in bioaugmented soil (diesel concentration - 15,000 mg/kg-soil and moisture content - 10%).

Fig. 2. Effect of indigenous microorganism on the mineralization of diesel in bioaugmented soil (NS: natural soil, BA: bioaugmentation, diesel concentration of 10,000 mg/kg-soil).
nearly flooded condition within the serum bottles during experiments. According to literature reports, high moisture content can significantly impair the soil aeration and can severely affect the microbial activity (Margesin and Scinner, 2005). According to Cho et al. (2000b), during chlorophenols bioremediation in soils, soil moisture (25–45%) had a significant negative effect on chlorophenols degradation and the rate of chlorophenol degradation was directly related to the soil moisture content. The authors attributed limited oxygen availability through soil agglomeration at 25% moisture content as the possible reason for a decrease in the degradation rate of chlorophenols. On the other hand, the effect of biomass dose on CO2 production showed about 48% increase in CO2 production (Fig 3c). Increased biomass dose might help in the adaptation of microorganisms within a very short time to the soil micro-ecosystem and prevent them from competing with the indigenous microorganisms.

According to Franco et al. (2014), CO2 production during diesel bioremediation showed a tendency to decrease depending on the number of experimental days. To assess CO2 production, samples of soil were contaminated with 0.5%, 2.0%, and 4.0% diesel (5, 20 or 40 g of diesel per kg of soil). According to the authors, although higher initial concentrations of diesel were releasing higher concentrations of CO2 after 30 and 60 days of treatment, the overall trend, which occurred in all samples, was a gradual reduction of CO2 production. However, during the end of the experiments, CO2 production was equal in all the tested samples (~450 ppm CO2) (Franco et al., 2014). The trend of CO2 production reduction was also confirmed by Mariano et al. (2007). In their experiments, the average CO2 production rate dropped to 34% in 20 days, which was presumably due to the depletion of carbon sources and a decrease in the bioavailability of diesel. As a survival strategy, the lack of easily accessible carbon sources forces microorganisms to switch over to more recalcitrant sources resulting in a noticeable drop of CO2 production (Mariano et al., 2007).

3.4. Effect of diesel concentration, moisture content and biomass dose on CO2 production

3.4.1. Regression analysis and ANOVA

In this study, the significance of the regression coefficients (at 95% confidence level) were determined using the Student’s ‘t’-test. In general, the larger the magnitude of ‘t’ and smaller the ‘p’, the more significant will be the corresponding coefficient term (Rene et al., 2007). The model presents an adjusted $R^2$ value of 0.998 which fitted the statistical model well.

The CO2 production (μmol) could be expressed by the following regression model equation.

$$\text{CO2 production (μmol)} = 2106.7 + 0.00730X_1 - 24.439X_2 + 3.2309X_3 + 0.000464X_1X_2 - 0.000059X_1X_3 - 0.09143X_2X_3 + 0.000003X_1X_2X_3 + 3546.82 Ct Pt$$

(3)

Generally, the results of regression analysis from this study showed that an increase in the diesel concentration and biomass dose had a positive effect on the CO2 production, while the effects were negative for moisture content. Among the linear effect terms, moisture content was found to have the highest linear negative effect ($t = -150.15$ and $p = 0.004$). Therefore, an increase of moisture content from its lower to higher value significantly decreased the CO2 production. As reported by Davis et al. (2003), moisture distribution in soils influences the removal of diesel in contaminated soils. Horel and Schiewer (2009) investigated the effect of moisture content on diesel removal in contaminated soils. The authors used different gravimetric moisture contents (2%, 4%, 8% and 12%) to diesel contaminated soil fertilized with 300 mg N/kg and incubated at 20 °C; however, on the contrary to other reports, the result of different treatments were considered to be insignificant in their study. A similar observation was also made by Ferguson et al. (2003), wherein the authors compared soils samples with 9.9%–39.8% gravimetric moisture contents and ascertained that the influence of moisture content was only minimal during the bioremediation of diesel contaminated soils.

Biomass dose showed the highest positive linear effect ($t = 133.15$ and $p = 0.001$) which corresponds to the increase of biomass dose from its lower to higher value. For diesel
concentrations, a positive correlation was observed when the diesel concentrations were increased from low to high levels (t = 35.98 and p = 0.018). As shown in several previous studies, elevated levels of initial biomass showed an increase in the pollutant removal rate as well as an overall improvement of the biodegradation process, since time required for the adaptation of microorganisms is reduced. For instance, Mishra et al. (2001) observed that the diesel removal efficiency after one year was significantly higher for soil samples inoculated with a microbial consortium (92%) compared to the removal for control samples (14%). Mukherjee and Bordoloi (2011) showed an improvement in TPH removal (76% after 180 days) after inoculation of selected microorganisms in comparison to non-inoculated control samples (3.6%). Similarly, in another report, the results of lab-scale experiments were confirmed by a larger scale field study by Szulc et al. (2014). However, in some studies, this strategy did not show any positive effect (Bouchez et al., 2000; Wagner-Döbler, 2003). Most likely, this failure can be attributed to the poor survivability of the introduced microorganisms as a result of improper strain selection (Szulc et al., 2014).

Concentrating interaction effects, the interactions between diesel concentration × moisture content (t = 27.26 and p = 0.023) and diesel concentration × biomass dose (t = 1.45 and p = 0.384) showed positive influence on CO2 production, while the interaction between moisture content × biomass dose (t = −55.97 and p = 0.011) showed a negative influence. Analysis of variance (ANOVA) was performed with nine degrees of freedom (Table 6) and the results showed that the linear effects (F = 13,856.24 and p = 0.006) of process parameters were statistically significant than the 2-way (F = 1292.77 and p = 0.020) and 3-way interactions (F = 209.47 and p = 0.044).

### 3.4.2. CO2 production from batch bioremediation experiments

The theoretical CO2 production during the bioremediation of diesel contaminated soil can be determined by the following stoichiometric equation:

\[
C_{24}H_{50} + 36.5 \text{O}_2 \rightarrow 24 \text{CO}_2 + 25 \text{H}_2\text{O}
\]

From Eq. (4), 1056 g of CO2 is expected to be produced during the aerobic oxidation of 338 g of diesel which corresponds to a theoretical CO2 production per unit amount of diesel as 3.1. CO2 production is usually ascertained as an indicator of bacterial respiration rate. Samples of CO2 (from different experimental runs) were collected once every two days. As seen from Fig 3, CO2 production started almost instantaneously during the first week, irrespective of the test conditions, and then the CO2 production started to stabilize and remain almost constant throughout the bioremediation process. Approximately 60% of the total CO2 was produced during the first eight days of bioremediation which could be attributed to the rapid mineralization of readily biodegradable fraction (linear and open chain hydrocarbons) of the diesel components (Atlas and Bartha, 1998).

The maximum and minimum CO2 production were found as 5729 µmol (252 mg) and 1512 µmol (66 mg) for experiments 9 (center point) and 3, respectively. This corresponds to a maximum CO2 production of 10.1 g/kg-soil at the center point and a minimum CO2 production of 2.7 g/kg-soil for experiment 3. Experiments carried out at the center point with moisture content of 20% (~60% field capacity) showed maximum CO2 production. These results are in agreement with literature results in which the optimum moisture content for stimulating the biodegradation of petroleum hydrocarbons in soil was shown to vary between 50% and 75% of the field capacity (Adams et al., 2011; Dibble and Bartha, 1979; Eweis et al., 1998). CO2 produced from the uncontaminated soil (soil background respiration) was 712.8 µmol, 1214 µmol and 244.4 µmol, respectively, for soil samples having a moisture content of 10%, 20% and 30%, respectively. In fixed bed bioreactors supplemented with nitrogen and phosphorous, Baptista et al. (2005) reported the best CO2 production per soil mass as 4000 mg CO2/Kg.

### 3.4.3. Main and interaction effects

The main (linear) and interaction effects of each independent parameter on CO2 production at 95% confidence level are shown in Fig. 4a and b, respectively. The main effects represent deviation of the average value between the high and low levels for each independent parameter. When the effect of the factors is positive, response value increases as the factor value changes from its low to high and negative effect occurs during the reverse phenomenon (Bingol et al., 2010; Mullai et al., 2010; Rene et al., 2006). The effects of diesel concentration and biomass dose were found to be positive. CO2 production was found to increase (2090–2264 µmol) when the concentration of diesel was increased from 5000 mg/kg-soil to 15,000 mg/kg-soil. This could be attributed to the fact that the increased diesel concentration could provide available organic source and the biochemical activity might increase along with the increase of hydrocarbon degrading microbial populations in the soil (Trindade et al., 2002). Nevertheless, it is noteworthy to also mention that toxicity effects could also increase during the microbial conversion of hydrocarbons due to the formation of acidic intermediates. Concerning biomass dose, CO2 production increased from 1854 to 2500 µmol when the biomass dose was increased from 50 mg/kg-soil to 500 mg/kg-soil. However, the main effect due to moisture content was found to cause a negative effect upon CO2 production, which was also corroborated by their negative Student’s ‘t’ test values (−150.15). The CO2 production decreased from −2541 to 1813 µmol when the moisture content was increased from 10% to 30%. Concerning centre point experiments (9 and 10), these operational conditions (Table 2) showed the highest CO2 production (5729 µmol) and were found to be suitable conditions for the diesel degrading microorganisms in the soil (data not shown), yielding a maximum diesel mineralization of −67%. Fig 5 shows the combined effects of biomass dose (BD), diesel concentration (DC) and moisture content (MC) on the mineralization of diesel (Dm, %). As seen from these profiles, increasing the values of process parameters from low to high levels caused an increase in the Dm values (rising ridge), up to a maximum (~70%) and a further increase in the values of process parameters decreased the Dm value. Based on the shape of these profiles, it can be concluded that there is relatively significant interaction (F = 1292.77, p = 0.020) between every two variables and the maximum predicted Dm were usually confined to the center point experimental values.

### Table 6

<table>
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<tr>
<th>Source</th>
<th>DF</th>
<th>Seq SS</th>
<th>Adj MS</th>
<th>F</th>
<th>p</th>
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<tr>
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<td>832,289</td>
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<td>0.005</td>
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<td>2-way interactions</td>
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<td>60,693</td>
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<tr>
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<td>99</td>
<td>2.11</td>
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<td>147,091</td>
<td>3133.05</td>
<td>0.011</td>
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<td>3-way interactions</td>
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<td>0.044</td>
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<td>X1 × X2 × X3</td>
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<td>9</td>
<td>22,271,379</td>
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Note.
DF: degrees of freedom; Seq SS: sequential sum of squares; Adj MS: adjusted mean of squares; F: Fischer’s variance ratio; p: probability value; X1: diesel concentration (DC, mg/kg-soil); X2: moisture content (MC, %); X3: biomass dose (BD, mg/kg-soil).
Fig. 4. Effects of diesel concentration (DC), moisture content (MC), and biomass dose (BD) on CO$_2$ production (µmol) in bioaugmented soil: (a) main effects, (b) interaction effects and (c) Pareto analysis.
Fig. 5. Effect of biomass dose (BD), diesel concentration (DC) and moisture content (MC) on the mineralization of diesel (Dm, %) in bioaugmented soil: (a) BD and DC, (b) BD and MC, and (c) DC and MC.
Concerning interaction effects, as shown in Fig 4b, moisture content × biomass dose showed the highest effect on CO₂ production which was also corroborated by ANOVA results (F = 3133.05, p = 0.011). As the value of the moisture content increased from 10% to 30%, and irrespective of the biomass dose, whether 50 or 500 mg/kg-soil, an antagonistic CO₂ production pattern was observed. Similarly, when the moisture content was increased from low to high levels, irrespective of the diesel concentrations (5000 or 15,000 mg/kg-soil), the CO₂ production reduced in all the test experiments. Evidently, all other interactions were found to result positive (synergistic) effect upon CO₂ production. Overall, in this study, we did not envisage two intersected lines to reveal substantial/strong interactions between the two factors. The observed trends were featuring almost parallel lines that showed almost negligible interaction between the two factors. Thus, it can be concluded that the interactions among the different independent parameters were found to be less significant compared to the main effects on CO₂ production.

The relative importance of main effects and their interactions on CO₂ production was represented by a Pareto chart (Fig 4c). At the 95% confidence level and with nine degrees of freedom, the F-value was calculated as 12.7. Those values that exceed the reference line (12.7) are statistically significant for CO₂ production. Among the main effects, moisture content showed the most significant effect on the cumulative CO₂ production followed by the biomass dose and lastly the diesel concentration.

4. Conclusions

The influence of process parameters, viz., diesel concentration, moisture content and biomass dose, on CO₂ production and diesel mineralization were investigated by performing statistically designed experiments. The results showed that diesel concentration and biomass dose showed synergistic influence on CO₂ production, while moisture content showed an antagonistic effect. Concerning the interaction effects, diesel concentration × moisture content and diesel concentration × biomass dose showed positive effects, while the interaction between moisture content × biomass dose was found to be negative. Results from ANOVA established that the overall linear effect was higher (F = 13,856.24 and p = 0.006) than the 2 and 3-way interaction terms. The maximum CO₂ production (~10,000 mg/kg-soil) was observed at the centre point conditions that yielded ~67% TPH mineralization. The proposed model equation will certainly provide a good estimation of CO₂ production in pilot-scale or field-scale systems, indicating the practical implications of applying statistical modeling for environmental bio remediation purposes.

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