Kinetics of Indigenous Isolated Bacteria used for \textit{ex situ} Bioremediation of Petroleum Contaminated Soil

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\textbf{Abstract:} The bioremediation of petroleum contaminated soil near to Jordan Petroleum Refinery was investigated using a laboratory scale aerated reactor. The Indigenous bacteria, \textit{Stenotrophomonas maltophilia}, were isolated from the contaminated sites and used further in the bioremediation experiments. First order kinetics is used in order to describe the rate of biodegradation. The results showed that the first order kinetic constants for the different bioreactors vary between 0.041 and 0.0071/day. The overall kinetic constant $k'$ was determined based on food-to-microorganisms ratio and found to be 0.02/day.

\textbf{Key words:} Bioremediation, kinetics, petroleum, soil, bioreactor

\section*{INTRODUCTION}

Remediation of contaminated soil has been considered as an important environmental topical study worldwide. Among the different remediation techniques, bioremediation has been proven to have the most effective approach to alleviate the environmental problems associated with contaminated soil [1].

Wide assortments of bacterial consortium, which are responsible for degradation of hydrocarbons found in petroleum contaminated soil are aerobic bacteria. The major microorganisms responsible for biodegradation of petroleum hydrocarbons have been found to be bacteria and fungi [2-4]. The genera to which hydrocarbon degrading bacteria belonged are \textit{Pseudomonas}, \textit{Alcaligenes}, \textit{Micrococcus}, \textit{Nocardia}, \textit{Corynobaeterium}, \textit{Rhodococcus}, \textit{Enterobacter}, \textit{Escherichia}, \textit{Arthenobacter}, \textit{Bacillus}, \textit{Streptomyces}, \textit{Clostridium}, and \textit{Proteus} [5].

Hwang \textit{et al}. [6] have investigated the bioremediation of hydrocarbon contaminated soil using composting process. They found out that mixing of remediated soil with contaminated soil has increased the effectiveness of composting. This is because the recycled soils usually have acclimated microorganisms that can significantly affect the degradation rate of contaminants.

Few works have been dedicated to investigate the kinetics of soil bioremediation [3, 6-11]. Information on kinetics is extremely important because it characterizes the concentration of the chemical remaining at any time and permits prediction of the levels likely to be present at some future time.

First-order kinetics is commonly used to describe biodegradation in environmental fate models because mathematically the expression can be incorporated easily into the models [8]. Many investigators grasp at first-order kinetics because of the ease of presenting and analyzing the data, the simplicity of plotting the log of the chemical remaining versus time as a straight line, and the ease of predicting future concentrations [7-9]. In different environments, first-order constants and the number of cells able to metabolize the substrate will differ [3, 8].

Hwang \textit{et al}. [6] investigated the bioremediation of diesel-contaminated using composting techniques. The results of the applied first order kinetics model agreed to a great extent with the experimental results. They found that the average first order kinetic rate constant of diesel oil was 0.099/day. Antizar-Ladislao \textit{et al}. [10] have studied the biodegradation of 16 polycyclic aromatic hydrocarbons using laboratory scale in-vessel composting at different temperatures. The degradation took place in mixed culture of bacteria, fungi, and actinomycetes. They found out that the first order kinetics can satisfactorily describe bioremediation process and the first order kinetic constant for all contaminants ranged between 0.009/day at 70°C and 0.013/day at 38°C. Li \textit{et al}. (2006) studied the biodegradation of diesel contaminated soil by an
isolated bacterial genus *Planococcus*. They used a Luong model to describe the bioreaction kinetics. The kinetic model was solved to obtain a maximum growth rate $\mu_{\text{max}} = 0.34/\text{h}$ and saturation concentration $K_s = 0.041\ \text{mM}$.

The primary objectives of this work are to isolate an indigenous bacterium capable to bioremediate petroleum contaminated soil and to find out the corresponding first order kinetic constant of the biodegradation process for the purpose of specific degradation rate determination.

**MATERIALS AND METHODS**

Soil samples were collected from three different petroleum contaminated sites near the Jordan Petroleum Refinery. Two samples were taken from each location at depths of 5 and ...??cm. Twenty grams of each soil sample was soaked in 100 mL of ringer’s solution (6.5 g NaCl, 0.42 g KCl, 0.25 g CaCl$_2$ per liter of distilled water) and shaken at 175 rpm for two hours [4]. Samples were then allowed to settle for 24 hours at room temperature. A volume of 5 mL of the supernatant was used to inoculate 45 mL of basal salt medium solution “BSM” (0.64 g K$_2$HPO$_4$, 0.31 g KH$_2$PO$_4$, 0.5 g NH$_4$Cl, 0.2 g MgSO$_4$, 7H$_2$O, 0.005 g FeSO$_4$, 7H$_2$O per liter of distilled water, pH 7) containing crude oil at concentration of 50 mg L$^{-1}$. Inoculated BSM samples were incubated at 37°C for 24 hours [12] and then 0.1 mL from the suspension was plated in duplicate on Petri dishes containing basal mineral salt medium (BSM agar). Cycloheximide (0.1 g L$^{-1}$) was added to the media in order to kill any possible existing fungi and to permit the growth of clear bacterial colonies. Petri dishes were incubated at 37°C for three days. After incubation, bacterial strain was isolated depending on the macroscopic characters of the colonies (shape, color, and size). The isolation was carried out by subculturing of each different colony on tryptose soy agar plates and incubating at 37°C for 24 to enhance the growth [13]. The isolated bacteria were cultivated in BSM solution at 37°C. This is considered as a bacterial stock that will be used as an inoculum in the further bioremediation experiments.

Identification of isolated bacteria was carried out according to the colony characteristics and using analytical profile index API 20 NE (bioMérieux) [14]. API 20 NE test is used as a bacterial species identification kit that encompasses 20 different biochemical tests. The results of the biochemical tests are then interpreted using bioMérieux specific code manual to identify the bacterial species.

Sandy soil samples from uncontaminated sites were collected and sterilized in an oven at 200°C for 48 h to remove any possible contaminant. Soil was then analyzed for organic matter to make sure that the soil contains no or negligible organic content. Crude oil from the Jordan Petroleum Refinery was mixed with 500 g of the sterilized soil at different ratios in sterilized glass bowl. Crude oil-soil mixture was placed in bioreactors constructed from 10 cm diameter PVC cylinder with working volume of 1.5 liter. A fine mesh was placed at the bottom of each bioreactor to allow blowing the compressed air into the oil-soil mixture. To ensure a moisture content of 20%, 10 mL of buffer BSM solution containing basic nutrient for bacterial growth was added daily by spraying over the soil surface and then soil was manually mixed. The bioreactors were inoculated by the isolated bacterial suspension at specific concentrations according to the specific experimental design. The concentration of microorganism in the bacterial suspension was measured using the gravimetric analysis of total suspended solid (TSS) at drying temperature of 105°C using moisture balance analyzer (Sartorius SMO 01).

A control bioreactor with all previous conditions but without bacterial inoculum was also used. Continuous oxygen supply was accomplished using air compressor. All experiments were conducted in an air-conditioned laboratory at an ambient temperature of around 20°C. Table 1 shows the setup summary of the entire experimental runs.

<table>
<thead>
<tr>
<th>Bioreactor*</th>
<th>Weight of soil (g)</th>
<th>Volume of oil (ml) **</th>
<th>Weight of indigenous isolated bacteria (mg)</th>
<th>Food-to-micro-organism ratio FM (mg TOC/mg TSS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>0</td>
<td>5</td>
<td>500</td>
<td>RIB1</td>
</tr>
<tr>
<td>0.5</td>
<td>20</td>
<td>5</td>
<td>500</td>
<td>RIB2</td>
</tr>
<tr>
<td>1.0</td>
<td>20</td>
<td>10</td>
<td>500</td>
<td>RIB3</td>
</tr>
<tr>
<td>-</td>
<td>0</td>
<td>50</td>
<td>500</td>
<td>R2B1</td>
</tr>
<tr>
<td>5.0</td>
<td>20</td>
<td>50</td>
<td>500</td>
<td>R2B2</td>
</tr>
<tr>
<td>2.0</td>
<td>50</td>
<td>50</td>
<td>500</td>
<td>R2B3</td>
</tr>
</tbody>
</table>

* stands for run, B stands for bioreactor. **Density of crude oil = 0.96 g cm$^{-3}$
Two soil samples of around 1 g were removed daily from each bioreactor to test for the rate of degradation. Crude oil in soil samples was extracted by adding 5 mL of dichloromethane (DCM) with vigorous shaking using vortex mixer for 5 minutes at 3000 rpm followed by centrifugation for 20 minutes at 2000 rpm. A sample of 500 μL was taken from the supernatant and mixed with 100 mL of 20% of sodium dodecyl sulfate (SDS), so that DCM-crude oil mixture could be solubilized in water. SDS-DCM-crude oil mixture was incubated in water bath at 40°C for 1 hour to evaporate DCM (boiling point of DCM is 39.7°C). After DCM evaporation, 30 mL of solution was taken and acidified (pH 2-3) by adding few drops of 10% H3PO4. Acidified sample was purged for 5 minutes using pure oxygen to strip out any inorganic carbons that might be present. Total organic carbon concentrations (TOC) in the acidified samples were then measured using Total Organic Carbon apparatus (Analytikjena Micro N/C).

RESULTS AND DISCUSSION

Bacterial isolation and identification: The result of Gram stain test showed that the isolated bacteria are Gram negative bacilli. The results of the biochemical tests incorporated in bacterial identification API 20 NE test according to API-bioMérieux standard procedure showed that the bacterial species is *Stenotrophomonas maltophilia* [15]. Similar results were found in different previous studies that were conducted on bioremediation of petroleum contaminated soils [4, 5, 16, 17].

Biodegradation kinetics: Microbial growth on pollutant mixture is an important aspect of bioremediation treatment. However, efforts to develop mathematical models for mixed substrate kinetics have been limited. When individual microbial species are considered, simple competition for the growth substrate is the only interaction included [9]. Here, our results are presented using *Stenotrophomonas maltophilia* growing individually on crude oil and compare mathematical models to describe these results.

The general formula of the first-order kinetic that can describe the rate of TOC reduction is:

\[
\frac{dC}{dt} = -kt
\]  

(1)

Where:

- t: time (day)
- C: remaining TOC concentration (mg L\(^{-1}\)) at any time
- k: first order kinetic constant (1/day)

In equation 1, it is assumed that the microbial concentration remains constant over the entire experimentation time. Therefore, the effect of microbial concentration on the kinetics constant is neglected.

The integration of equation 1 leads to the known formula of the first-order kinetics

\[
C = C_0 e^{-kt}
\]  

(2)

Where \(C_0\) is the initial TOC concentration (mg L\(^{-1}\)).

\[
\ln\frac{C}{C_0} = kt
\]  

(3)

In order to experimentally calculate the kinetic constant \(k\), equation 2 is linearized using the following equation

The first-order kinetics is said to be valid if a linear relationship is achieved upon plotting the logarithmic part of equation 3 versus time. Analysis of the rates of hydrocarbons removal showed that most compounds obeyed first-order kinetics [8]. The slope of the line represents the first-order kinetic constant \(k\).

The linearization results of the experimental data are graphically presented in Fig. 1-4. The kinetic constants for all runs calculated from the slopes of the linearization are summarized in Table 2.

The results in Table 2 show that the first order kinetic constants for the different bioreactors at different food-to-microorganisms ratios range between 0.0071 and 0.041/day. These results agree in a great extent with the results achieved in previous studies [6, 7, 10].

The validation of the first order kinetics was tested using the experimental results that were also reported in previous study [15]. The model validation results are graphically presented in Fig. 5-8. These figures show that the first order kinetic can be used with relatively considerable accuracy in order to describe the bioremediation process of petroleum contaminated soil. In contrary to the assumption made in equation 1, which states that the effect of microbial concentration on kinetic constants is neglected, Table 2 shows, that

| Table 2: First-order kinetic constants of three experimental runs |
|-----------------|-----------------|-----------------|-----------------|
| Experiment     | F/M ratio (mg TOC/mg TSS) | Kinetic constant (k) (day\(^{-1}\)) | R\(^2\) |
| R1B2           | 0.5             | 0.041           | 0.92           |
| R1B3           | 1.0             | 0.019           | 0.84           |
| R2B3           | 2.0             | 0.015           | 0.80           |
| R2B2           | 5.0             | 0.007           | 0.71           |
the kinetic constant could be related to the microorganism concentration (food-to-microorganisms ratios, F/M). For a comprehensible relationship between these two parameters, the kinetic constant (k) is plotted against the reciprocal F/M ratio as shown in Fig. 9.

Table 2 and Fig. 9 show clearly, that the value of kinetic constant is greatly dependent on the microorganism concentration, so that an increase in microorganism concentration (a decrease in the reciprocal F/M ratio) results in an increase in kinetic constant value. The equation that represents the linear relationship between the k values and the reciprocal F/M ratios is determined by using linear regression. The regression result is summarized in equation 4.

\[
k = \frac{k'}{F/M}
\]  

(4)

Where \( k' \) is the linearization constant found in Fig. 9 which is equal to 0.02/day. This represents the overall kinetic constant. Equation 5 is resulted from substitution of equation 4 in equation 2:

\[
C = C_0 \cdot e^{-k/F/M}
\]  

(5)

This equation represents the overall kinetic equation for the bioremediation of crude oil contaminated soil using aerobic \( S\textit{trenotrophomonas maltophilia} \) bacteria. The first-order rate kinetic constants found in this work are similar to that found in different previous studies. Hutchins \textit{et al.} [7] reported that first-order rate constant ranged from a low of 0.016 to a high of 0.38/day depending on compounds of hydrocarbon. Hwang \textit{et al.} [6] found that the first order kinetic constant of diesel oil was 0.099/day. Antizar-Ladislao \textit{et al.} [10] showed that the first order kinetic constant at 38°C was 0.013/day.

REFERENCES


