Kinetics of crude oil degradation by an indigenous mixed culture isolated from palm oil mill effluent

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Abstract

Objectives: To investigate the microbial growth kinetics and degradation of crude oil (CO) by mixed cultures isolated from palm oil mill effluent (POME).

Methods/Statistical analysis: The scanning electron microscope was employed to examine the surface morphology of POME and the CO contaminated soil. Microbial count using the total viable count (TVC), mold, and coliform counts were investigated at different levels of CO contamination. Findings: The TVCs at 20, 40, and 60 mg/L of CO were 7.3x10^7 CFU/g, 6.8x10^6 CFU/g and 7.2 x 10^6 CFU/g, respectively. The concentration of the CO was utilized at 20 mg/L indicating that this concentration did not inhibit microbial growth. Based on the correlation coefficient (R^2) values obtained for the four growth kinetic models, the Haldane model with R^2 of 0.97 gave the best fit. Application/Improvements: Finally, the one way ANOVA and Tukey's honest significant difference (HSD) indicated the statistically significant effect (P< 0.05) of the initial CO concentration on microbial growth.

Keywords: Kinetics; Degradation; Mixed culture; Crude oil; Palm oil mill effluent

1 Introduction

The incident of the spill from leakage pipes, storage tanks and lack of safety measures during the drilling of crude oil (CO) are some of the many ways by which crude oil gets in contact with the soil and causes environmental pollution(1). CO and other xenobiotic contaminants are generally referred to as toxic hydrocarbon compounds and are toxic to soil microorganisms and plants(2). Thus, the elimination of CO from the soil is necessary to preserve environmental quality(3). However, biological treatment of CO has been proven to be economical and an easily manage approach towards CO degradation from the environment(4). Also, the microbial population could have a considerable prospect to remedy hydrocarbon contaminated soil. Previous works on the isolation of hydrocarbon degrading microbial culture indicated that a consortium of hydrocarbon degraders with better degrading properties was more beneficial(4). However, reports that the mixed cultures of Pseudomonas aeruginosa and Bacillus subtilis were
able to degrade 250mg/L of phenol and wattle completely within 36 and 48 hours, respectively. In this present study, we investigated the growth kinetics and degradation capabilities of mixed microbial culture isolated from palm oil mill effluent (POME) on crude oil degradation. Microbial counts in the palm oil mill effluent (POME) and CO contaminated soil were estimated using the total viable count (TVC), mold, and coliform counts. The one-way analysis of variance (ANOVA) and Tukey’s post-hoc multiple comparison tests was used to ascertain the statistical significance (<0.05) effect of crude oil concentration on microbial growth. Furthermore, the surface morphologies of the raw samples of POME and crude oil contaminated soil were studied using the scanning electron microscope (SEM).

2 Materials and Methods

2.1 Samples collection and preparation

The crude oil (CO) was obtained from the Nigerian National Petroleum Cooperation (NNPC) located in Port Harcourt Rivers State Nigeria. The palm oil mill effluent was obtained from a palm oil milling factory located in Ogwofia Ozom Mgbabu-owa Enugu State Nigeria. Prior to the soil contamination with CO, the soil was obtained from the botanical garden of the University Of Nigeria Nsukka Enugu State.

2.2 Microbial count (total viable count, mold and coliform counts)

1 g of the sample was placed into 9 ml of Ringer solution containing (g/mL): 6.5 NaCl, 0.25 CaCl, and 0.2 NaHCO₃. The mixtures were mixed thoroughly on a rotary shaker at 150 rev min⁻¹. The mixture was further diluted by transferring 1 mL into three test tubes containing 9 mL Ringer solution using a sterile pipette. Then 0.1 mL of the diluted solution was transferred from each dilution test tube into three sterile Petri dishes using a sterile pipette. For Total viable count, (TVC) mold count, and coliform count, 15 ml each of sterile nutrient agar, Sabouraud Dextrose Agar (SDA) and sterile violet red bile agar, respectively was poured into each of the Petri dishes and gently rotated on the table to mix. The nutrient agar, SDA and sterile violet red bile agar in the Petri dishes were allowed to gel for 10 min before sealing and was incubated in an inverted position at 37°C for 24 hours. The colonies formed were counted using Quebec colony counter and expressed in colony forming unit per gram (CFU/g).

2.3 Isolation of CO degrading microorganism from POME

Nutrient agar and broth purchased from Oxoid, Basingstoke UK were used for plating and culturing the microbial strain, respectively. The mixed culture was prepared by growing them on a malt extract agar plates at 25°C for 30 days in the dark. In order to investigate the ability of the mixed microbial culture to degrade CO, the culture was grown on a 250 mL Erlenmeyer flask containing 50 mL of mineral salt medium (MSM). The MSM had the following compositions (g/L): 0.01 (NH₄)₂SO₄, 0.2 NH₄Cl, 0.25 K₂HPO₄, 0.02 MnSO₄, 0.5 MgSO₄·7H₂O, and 0.01 CaCl₂. The pH was maintained at 7.0±0.2. The MSM containing CO as a sole carbon source (6) was incubated for 30 days on a rotary shaker (VXR, Vibrax shaker, 220V, 50/60Hz) set at 150 rpm and 37°C. This was in order to obtain cultures with a stable CO degrading ability.

2.4 Crude oil degradation procedure

The batch degradation of crude oil (CO) was conducted in a 500mL Erlenmeyer flask containing 200g of the CO contaminated soil with 100ml of sterile MSM. The Erlenmeyer flasks contained different initial CO concentrations (20, 40, and 60 mg/L). Prior to the inoculation of the isolated culture from POME into the flasks containing the contaminated soil, the culture was transferred into 100mL of the MSM containing CO and grown at 27°C in an incubator while the shaking speed was maintained at 150rev/min.

2.5 Extraction of residual CO

Soxhlet extraction process was used to extract 5mL of the liquid culture using acetone/dichloromethane (1:1v/v) as the solvent. After extraction, the solvent was left to evaporate and the remaining residue was dissolved in 5mL of dichloromethane. The CO concentration was estimated using a gas chromatography coupled with a mass spectrometer (GC-MS Shimadzu QP-500). The carrier gas was helium (purity > 96.9%) at a constant flow rate of 1.5 mL min⁻¹ with column pressure at 100 kpa. The injection volume and temperature were 1 µL and 80°C, respectively. The concentrations of CO were determined after calibrations of the method with standard chrysene samples at different concentrations. CO degradation was calculated using Eqn. (1).

\[
\%\text{CO degradation} = \frac{\text{initial CO concentration} - \text{final CO concentration}}{\text{initial CO concentration}} \times 100
\] (1)
2.6 Statistical Analysis

One way analysis of variance (ANOVA) and Tukey's honest significant difference (HSD) was processed using IBM SPSS statistical package (version 22). Tukey's HSD test predicts the honest significant difference that must exist in distance between two groups mean. The statistical significance of the data analysis was set at $P < 0.05$. The analysis was used to investigate the statistical significance of CO initial concentration on microbial growth.

2.7 SEM Analysis

The samples were analyzed using SEM (ASPEX 3020) at 10 Kv to determine their morphological characteristics. The samples were centrifuged, dried at 110°C for 4 hours and coated with a gold film in a sputter coater before the examination.

3 Results and Discussion

3.1 SEM Analysis for POME and CO contaminated soil.

The SEM micrographs for the crude oil contaminated soil and POME is shown in Figures 1 and 2, respectively.

![SEM image for CO contaminated soil](https://www.indjst.org/)

The micrographs of POME in Figure 2 showed a dotted surface morphology, which was attributed to the oily nature of the POME used for the SEM analysis. However, the surfaces of the CO contaminated soil and POME was rough and porous, respectively. The levels of porosity observed the CO contaminated soil and POME showed that the CO contaminated soil was more porous (Figure 1). The rough surface of the CO contaminated soil indicated that soil topography and texture may have been altered after the CO contamination. The CO may have penetrated the closely packed inner layer of the soil thereby enlarging the CO contaminated soil (Figure 1).

3.2 Microbial Count

Microbial populations in POME and the CO contaminated soils were estimated using the Total viable count (TVC), mold, and coliform count (Table 1). The TVC is the total viable microbial population while the coliform and mold were the active bacteria and filamentous fungi present in the samples, respectively.

The colony forming unit per gram (CFU/g) of molds in POME was in the magnitude of $6.3 \times 10^7$, indicating a higher level of molds in POME in comparison with the TVC ($4.1 \times 10^6$ CFU/g) and coliform counts ($3.1 \times 10^4$ CFU/g) (Table 1). These results
suggest that the predominant microbial population in POME was mold. The pH is another important factor that affects soil microbial population[7]. The slightly acidic pH (4 to 5), of POME as reported previously[8–10] could be contributing to the increased CFU/g of molds, as molds generally grow well in acidic condition[11].

The naturally occurring microbial population in the soil prior to CO contamination in terms of the TVC coliform and mold count showed that the soil contained a higher level of TVC (7.3 x 10^6 CFU/g). The mold and coliform counts were relatively close in the order of 4.3 x 10^6 CFU/g and 4.1 x 10^5 CFU/g, respectively. These results indicated how rapidly the microbial population adapted to the soil (Table 1).

However, one of CO's indirect effects on soil was attributed to the changes in the microbial population[12]. However, the changes in the microbial counts in the CO contaminated soil could be stimulatory or inhibitory to soil microorganisms. The effects of CO concentrations on soil microbial count (TVC, mold and coliform count), is presented in Table 2. It was observed that the addition of CO to the soil increased the TVC of the microbial population as the TVCs at 20, 40, and 60 mg/L of CO were 7.3 x 10^7 CFU/g, 6.8 x 10^6 CFU/g and 7.2 x 10^6 CFU/g, respectively (Table 2). These observations suggest that the CO within the concentrations used in this study could be stimulatory and also be acting as a source of carbon for the growth of the indigenous microbial population[5]. However, the raw CO used in this study may be less volatile to atmospheric losses thereby increasing their bioavailability as a carbon source for the soil microbial population.

3.3 Effects of CO initial concentration on microbial growth

The results from the effect of initial CO concentration on microbial growth are shown in Figure 3. The log CFU/g of soil sample was observed to be high at 20 mg/L initial CO concentration. The high log CFU/g (4.3 x 10^6 CFU/g) observed at 20
Table 2. Microbial counts at different CO concentration in CO contaminated soil

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CO concentrations (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
</tr>
<tr>
<td>TVC (CFU/g)</td>
<td>$7.3 \times 10^7$</td>
</tr>
<tr>
<td>Coliform (CFU/g)</td>
<td>$5.2 \times 10^3$</td>
</tr>
<tr>
<td>Mold count (CFU/g)</td>
<td>$7.0 \times 10^5$</td>
</tr>
</tbody>
</table>

TVC; Total viable count of microorganism, CFU/g; colony forming unit per gram.

mg/L could be due to the acclimatization period (one month), which was favorable at 20 mg/L. The CO could serve as a carbon source, but at extreme concentration can be toxic to microbial growth. However, previous studies reported the importance of carbon to microbial growth and synthesis (13). The CO degradation could be successful at 20 mg/L as high log colony forming unit (CFU) of microorganism per gram of soil was obtained. Consequently, the available carbon at 20 mg/L might be supplying the needed carbon and energy for microbial growth (6,13). However, the log CFU/g at 40 mg/L ($2.12 \times 10^6$ CFU/g) was observed to be low indicating a reduced CO degradation at this concentration (14,15). The low log CFU/g in the control sample indicated that the presence of the indigenous microorganism in the uncontaminated soil. The available microorganism in the control sample might not be suitable for CO degradation as the microorganisms were not acclimatized in the presence of CO (Figure 3).

3.4 One way ANOVA for the effect of initial CO concentration on microbial growth

Considering the independent variable of initial CO concentration, one way ANOVA was used to judge the statistical significance (P<0.05) of this variable on microbial growth. The null hypothesis under investigation suggests that the mean number of microbial growth is the same at all initial CO concentration. The P-value, in Table 3 was used to decide the conclusion of the investigation. It was evident from Table 3 the mean effect of the initial CO concentration on microbial growth was statistically significant with F statistics of 721 and P < 0.0001. However, based on the one way ANOVA results in Table 3, the null hypothesis was rejected.

Table 3. ANOVA for the effect of initial CO concentration microbial growth

<table>
<thead>
<tr>
<th></th>
<th>Sum of Squares</th>
<th>df</th>
<th>Mean Square</th>
<th>F</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between Groups</td>
<td>240.333</td>
<td>2</td>
<td>120.167</td>
<td>721</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Within Groups</td>
<td>0.500</td>
<td>3</td>
<td>0.167</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>240.833</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The Tukey’s honest significant difference (HSD) test explores the statistical significance among the group means in order to
provide specific information on which means differed. From the Tukey’s HSD test in Table 4, statistically, significant means were identified with asterisks (*). However, these means correspond to statistically significant P-values (P < 0.0001). Accordingly, the HSD test in Table 4 shows that the mean difference (I-J) between the group means at 20 mg/L and 60 mg/L were statistically significant (P < 0.0001) whereas, the mean difference (I-J) at 40 mg/L was not statistically significant (P < 0.05). Also, a nonzero difference in mean was probably unlikely due to the non-zero 95% confidence level (Table 4).

**Table 4.** Tukey post-hoc (HSD) analysis for the effect of initial CO concentration

<table>
<thead>
<tr>
<th>(I) concentration</th>
<th>(J) concentration</th>
<th>Mean Difference (I-J)</th>
<th>Std. Error</th>
<th>P-values</th>
<th>95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mg/L</td>
<td>40 mg/L</td>
<td>-15.500*</td>
<td>0.408</td>
<td>0.000</td>
<td>-17.21 -13.79</td>
</tr>
<tr>
<td>20 mg/L</td>
<td>60 mg/L</td>
<td>-8.000*</td>
<td>0.408</td>
<td>0.001</td>
<td>-9.71 -6.29</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>20 mg/L</td>
<td>15.500</td>
<td>0.408</td>
<td>0.802</td>
<td>13.79 17.21</td>
</tr>
<tr>
<td>40 mg/L</td>
<td>60 mg/L</td>
<td>7.500</td>
<td>0.408</td>
<td>0.550</td>
<td>5.79 9.21</td>
</tr>
<tr>
<td>60 mg/L</td>
<td>20 mg/L</td>
<td>8.000*</td>
<td>0.408</td>
<td>0.001</td>
<td>6.29 9.71</td>
</tr>
<tr>
<td>60 mg/L</td>
<td>40 mg/L</td>
<td>-7.500*</td>
<td>0.408</td>
<td>0.001</td>
<td>-9.21 -5.79</td>
</tr>
</tbody>
</table>

* The mean difference is significant at P-values less than 0.05

3.5 Kinetics of microbial growth

Microbial growth kinetic models in Table 5 were fitted to the experimental data at varying initial CO concentration. In order to determine the microbial growth parameters, experimental values of the specific growth rate (μ) were estimated using the expression in Eq. (2) where Xₜ and X₀ are the final and initial microbial concentration, respectively, t is the time (hrs) (16),

\[ X_t = X_0e^{\mu t} \]  

The obtained values of μ were used to estimate the kinetic parameters for Monod, Haldane, and Verhulst models using the nonlinear regressions according to (16). From Tables 5 and 6, \( \mu_{max} \) (hr⁻¹) is the maximum specific growth rate, \( K_i \) (mg/L) is the CO inhibition constant, \( K_s \) is the CO affinity constant, \( X_m \) (mg/L) is the maximum microbial cell concentration and S (mg/L) is the CO concentration (17).

**Table 5.** Nonlinear microbial growth kinetics models

<table>
<thead>
<tr>
<th>Growth models</th>
<th>Nonlinear</th>
<th>Plots</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monod</td>
<td>( \mu = \frac{\mu_{max}S}{K_s + S} )</td>
<td>( \mu ) vs S</td>
<td>(18)</td>
</tr>
<tr>
<td>Haldane</td>
<td>( \mu = \frac{\mu_{max}S}{K_s + S + S^2} )</td>
<td>( \mu ) vs ( S^2 )</td>
<td>(17)</td>
</tr>
<tr>
<td>Verhulst</td>
<td>( \mu = \mu_{max}(1 - \frac{X_t}{X_m}) )</td>
<td>( \mu ) vs ( X_t )</td>
<td>(18)</td>
</tr>
</tbody>
</table>

Figure 4, showed the nonlinear plots of specific growth rate (μ) versus CO concentrations. It could be observed that the μ of the indigenous microorganisms increased with an increase in the initial CO concentration as no lag phase was observed. This result indicated a good adaptation of the indigenous microbial population towards the CO contaminated soil (18,19).

According to Figure 4, the μ increased at 20 mg/L after which, a slight decline was observed. The increase in the μ was mainly during the exponential phase when the microbial cells are actively growing. Another reason for the observed increase in μ at low CO concentration could be that at 20 mg/L, the effect of CO inhibition on indigenous microbial population was negligible. However, at low contaminant concentrations, (20) reported that the effect of substrate toxicity was negligible in microbial metabolic activity.

From the \( K_i \) values presented in Table 6, It was observed that the \( K_i \) values increased with an increase in CO concentrations as the lowest \( K_i \) values were obtained at 20 mg/L. However, (21,22) noted that the low \( K_i \) value implies a higher affinity for the substrate while a high \( K_i \) value indicated a lower affinity for the substrate. This indicated that the indigenous microbial population had a high affinity at low CO concentration (20 mg/L).

The maximum microbial concentration (\( X_m \)) values were also calculated using the Verhulst model and presented in Table 6. The results showed that the \( X_m \) values at low CO concentration were high. For example, the \( X_m \) at 20 mg/L initial CO concentration was 12.17 mg/L, whereas, it was 3.0 mg/L at the initial CO concentration of 60 mg/L. However, the \( K_i \) values inherent
Fig 4. Non-linear plot of specific growth rate versus CO concentrations

Table 6. Microbial growth kinetic parameters

<table>
<thead>
<tr>
<th>Initial CO conc. (mg/L)</th>
<th>Monod</th>
<th>Haldane</th>
<th>Verhulst</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\mu_{\text{max}} = 0.0481$</td>
<td>$\mu_{\text{max}} = 0.0401$</td>
<td>$\mu_{\text{max}} = 0.0249$</td>
</tr>
<tr>
<td></td>
<td>$K_s = 5.11$</td>
<td>$K_s = 1.9$</td>
<td>$X_m = 12.17$</td>
</tr>
<tr>
<td></td>
<td>$R^2 = 0.903$</td>
<td>$K_s = 28.9$</td>
<td>$R^2 = 0.831$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$R^2 = 0.989$</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>$\mu_{\text{max}} = 0.0439$</td>
<td>$\mu_{\text{max}} = 0.0401$</td>
<td>$\mu_{\text{max}} = 0.0320$</td>
</tr>
<tr>
<td></td>
<td>$K_s = 15.2$</td>
<td>$K_s = 35.39$</td>
<td>$X_m = 10.01$</td>
</tr>
<tr>
<td></td>
<td>$R^2 = 0.892$</td>
<td>$K_s = 2.3$</td>
<td>$R^2 = 0.843$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$R^2 = 0.992$</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>$\mu_{\text{max}} = 0.0362$</td>
<td>$\mu_{\text{max}} = 0.0332$</td>
<td>$\mu_{\text{max}} = 0.0315$</td>
</tr>
<tr>
<td></td>
<td>$K_s = 18.92$</td>
<td>$K_s = 37.22$</td>
<td>$X_m = 6.2$</td>
</tr>
<tr>
<td></td>
<td>$R^2 = 0.821$</td>
<td>$K_s = 5.3$</td>
<td>$R^2 = 0.832$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$R^2 = 0.988$</td>
<td></td>
</tr>
</tbody>
</table>

to the Haldane model increases with an increase in CO concentration indicating that the effect of CO inhibition was negligible at low CO concentrations,\(^{(23,24)}\). However, the $R^2$ was frequently used to judge whether the studied model represents correctly the experimental data,\(^{(25–27)}\). Among the microbial growth models studied the Haldane inhibition growth model showed the best fit of the experimental data ($R^2$ greater than 0.95) at all initial CO concentration as compared to Monod and Verhulst models ($R^2$ less than 0.95) Table 6.

4 Conclusions

It can be concluded that mixed microbial culture isolated from palm oil mill effluent (POME) was able to degrade crude oil (CO) at 20 mg/L initial concentration. The growth kinetic parameters from the microbial growth models show the intrinsic behavior of the mixed cultures towards the different levels of CO. The statistically significant effect ($p < 0.05$) of the CO concentration on microbial growth was evident according to the one way ANOVA and Tukey’s post-hoc HSD tests. However, the Haldane model gave the best fit for the experimental data on specific growth rate at all CO concentration ($R^2$ greater than 0.95). The capability of the mixed culture to degrade CO shows the benefits of mixed cultures in hydrocarbon degradation.

https://www.indjst.org/
References


11) America Industrial Hygiene Association AIHA; Standard Methods for the Examination of mold count in Water and Wastewater. 2011.


