Degradation of Hydrocarbons and Lignin-like compounds by *Alcaligenes* sp. strain 3k isolated from Ilorin

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**ABSTRACT:** The primary goal of this study was to isolate hydrocarbon-degrading organisms and assess their ability to bioremediate petroleum-contaminated soil and water. Nigeria is one of the major oil producing countries and petroleum contamination is widespread in agricultural soil. *Alcaligenes* sp. strain 3k was isolated from a kerosene-polluted soil in Ilorin, Nigeria. We also assessed its ability to degrade plant lignin, as lignin is a complex aromatic heteropolymer commonly found in soil and aquifer environment. Strain 3k was originally grown on mineral salts medium with kerosene as a sole energy and carbon source. The capacity of the isolate to degrade both aromatic, aliphatic hydrocarbons and lignin-like compounds was tested. Among the tested compounds, the organism utilized kerosene, hexadecane, cyclohexane, phenol and benzoate as the sole sources of carbon. In addition, strain 3k also degraded various lignocellulose compounds as the sole source of carbon. However, hexane, benzene, toluene, ethylbenzene and xylene were not metabolized. Our study demonstrates that soil organisms like *Alcaligenes* could play important role in the reclamation of petroleum-contaminated soil and water. Utilization capacity of lignin as the sole carbon source suggest that these organisms can survive on plant detritus and also have the ability to degrade hydrocarbons upon accidental or deliberate contamination of agricultural soil and water.

**Keywords:** *Alcaligenes* sp; hydrocarbons; lignin-like; aromatics; heteropolymer.

**INTRODUCTION**

Nigeria has Africa’s largest reserves of oil and gas resources, mostly in the Niger Delta and on the continental shelf of the country. Oil extraction in the Niger Delta has been going on since the 1950s and Nigeria exports a significant amount of oil every day. Oil spills have occurred repeatedly for decades in the Niger Delta and large parts of the land and wetlands are intermittently and chronically affected by oil spills that destroy crops and aquaculture through the contamination. There is also rampant mismanagement of the land resources. These have significant negative consequences on crop yield and land productivity, which further impoverish the already poor farmers in these areas. Cost of farm produce also increases due to shortage of supply. With the increasing soil infertility because of the destruction of soil microorganisms and dwindling agricultural productivity, farmers have been forced to abandon their land to seek non-existent alternative means of livelihood.
Kerosene is a colourless flammable hydrocarbon liquid obtained from the fractional distillation of petroleum at 150°C and 275°C (Gouda et al., 2008). Despite the several usefulness of kerosene, it also constitutes a major environmental concern worldwide (Saratale et al., 2007). Kerosene is not considered a carcinogen but repeated exposure of animals to kerosene has been linked to skin cancer (Chilcott, 2006). In addition to aesthetic problem and economic damage caused by kerosene spills, crops and animals in both land and water are affected negatively (Ikpeme et al., 2007). Microorganisms with the ability to degrade oil are ubiquitously distributed in soil and marine environments (Cao et al. 2009; Harayama et al. 2004; Head et al. 2006; Jyothi et al. 2012; Marques-Rocha et al. 2000). Degradation of Jet fuel by Gram-negative bacteria isolated from kerosene-polluted soil had been reported previously (Adetitun et al., 2018).

The degradation rates of hydrocarbons are affected by several chemical, bio-physical parameters such as pH, temperature, nutrient and quantity of hydrocarbons (Santhini et al. 2009). Alkanes vary in chain length from one in methane to greater than 48 carbon atoms. They are the major components of petroleum and petroleum products and make up about 20-50% of crude oil (So & Young, 1999). Several microorganisms (Rojo, 2009) can efficiently degrade most alkanes. Aerobic degradation of alkanes begins with oxidation of one of the methyl groups (either terminal or subterminal) to produce the corresponding primary alcohol by the enzyme alkane hydroxylases. The alcohol is finally converted to a fatty acid (van Beilen et al. 2003; Ji et al. 2013).

Benzoate has been widely used as a prototype compound for the study of the bacterial catabolism of aromatic compounds (Gibson & Harwood, 2002; Carmona et al. 2009). The monoaromatic hydrocarbons such as benzene, ethylbenzene, toluene and o-, m-, and p-xylene (BTEX) are constituents of petroleum and its products such as gasoline and diesel fuel. (Dean, 1985). BTEX compounds are toxic and carcinogenic and difficult to degrade, especially under anaerobic conditions (Phelps & Young, 1999).

Lignin is a complex heteropolymer comprised of a network of aromatic phenypropanoid units linked by C–C and C–O–C binding (Brown & Chang 2014; Ralph et al. 2004). Microorganisms have evolved the capacity to degrade lignin to gain access to plant polysaccharides to meet their metabolic needs. The exploitation of this capacity offers a natural means for preparing plant biomass for biofuels production (Brown & Chang 2014; Bugg & Rahmanpour, 2015; Mosier et al. 2005). It is known that many fungi and bacteria produce lignin degrading enzymes such as peroxidases and laccases (Bugg et al. 2011a, b; Kumar et al. 2009; Martinez, et al. 2009) that not only depolymerize or degrade lignin, but also degrade petroleum hydrocarbons (Falade et al. 2017; Marco-Urrea et al. 2012). Lignin is a major component of soil organic matter (Datta et al. 2017) that can support soil microbial community including hydrocarbon-degrading organisms. This is important as these organisms can quickly degrade hydrocarbons upon sudden contamination of soil and water by petroleum compounds. In addition, these organisms can play important role in carbon cycling and delignification of plant biomass for improved biofuel production (Brown & Chang 2014; Bugg & Rahmanpour, 2015; Mosier et al. 2005). Puentes-Téllez & Salles (2018) reported that cellulose and hemicellulose degradation were either negatively or not affected by functional diversity whereas functional diversity positively correlated with the breakdown of lignin.

The present work aims at characterization of Alcaligenes sp. strain 3k and assessing its degradation of hydrocarbons and lignin-like compounds in vitro with the hope that the
same results obtained can be replicated on non-experimental samples.

**MATERIALS AND METHODS**

Hexadecane, hexane, cyclohexane, benzoate, phenol, benzene, toluene, ethylbenzene and xylene used in this study were obtained from Sigma-Aldrich (St Louis, MO, USA). Also, lignocellulose such as vanillic acid, vanillin, veratric acid, methylvanillin, syringic acid, cinnamic acid, anisoin (4,4-dimethoxybenzoin), o-benzylvanillin (4-benzylxy-3-methoxy-benzoaldehyde), alkali lignin, and cellulose were purchased from Sigma-Aldrich. Xylan (birch wood) was purchased from Fluka. Most of the reagents used in this study were of reagent grades.

Kerosene-metabolizing microorganisms were briefly enriched in mineral salts medium (Dalvi et al. 2016) containing kerosene as the carbon source from alfisloam artificially contaminated with kerosene at the University Of Ilorin, Nigeria. Pure cultures were isolated by 10-x dilution of the enrichment culture and spreading 0.1-ml aliquots of the diluted culture onto agar plates. Plates were incubated at 30 °C and well-isolated colonies were re-streaked three more times on new plates prior to transferring into 250-ml conical flasks containing 100 ml of mineral salts medium (MSM) with 1 ml of kerosene. The isolate was identified as a species of the genera Alcaligenes closely related (99 % similarity) to Alcaligenes faecalis using PCR. Genomic DNA was isolated using a commercially available kit (FastDNA spin kit for soil, MP Biomedicals, Santa Ana, CA). 16S rRNA-genes were amplified with 27F and 1492R primers as described before (Spear et al., 2005). Amplified PCR products were cleaned using ExoSAP-IT and sequenced at the DNA core facility, Oklahoma State University, Stillwater, OK. Amplified 16S rRNA-gene sequence was analyzed using the National Center for Biotechnology Information (NCBI) database using BLASTn.

Degradation of hexane, hexadecane, cyclohexane by strain 3k was carried out in flasks containing 50 ml of MSM supplemented with 24.7 mM hexane (65 μL neat hexane), 11 mM hexadecane (65 μL neat hexadecane), or cyclohexane 30.05 mM (65 μL neat cyclohexane) as the sole source of carbon. Bottles were inoculated with roughly 10^4 to 10^5 cells/ml and incubated at 30°C static in the dark. Degradation of hydrocarbons was monitored by determining the growth of the organism. Culture sample (1 mL) was withdrawn periodically from each flask and plated on to LB plates. Colony forming units (CFU) was determined.

Degradation of benzoate and phenol was carried out in 50 mL tubes containing 25 ml of MSM with 2 mM sodium benzoate or phenol as the sole carbon source. Tubes were inoculated with 10^3 cells/ml of strain 3k and incubated static at 30°C in the dark. Un-inoculated control tubes with benzoate or phenol were set up similarly. Culture sample (1 mL) was withdrawn from each tube and the concentration of benzoate and phenol was determined by UV absorption at 223 nm and 269 nm, respectively using a spectrophotometer. The spectrophotometer was blanked with distilled water.

Utilization of BTEX by the isolate was carried out in 110 mL of serum bottles containing 50 mL of MSM with 28 μ mole benzene (2.5 μL neat), 23.85 μ mole toluene (2.5 μL neat), 20.4 μ mole ethylbenzene (2.5 μL neat) and 20.35 μ mole xylene (2.5 μL neat). Bottles were capped with Teflon-coated septa and aluminum crimps. For each compound, three live and two autoclaved control bottles were setup. Headspace samples (100 μL) was withdrawn periodically and analyzed using a gas chromatograph using the methods described elsewhere (Nicholson & Fathepure, 2004).

Utilization of lignin monomers and dimers as well plant polysaccharides (xylan
and cellulose) was carried out in 250 ml flasks containing 100 ml of MSM and 0.02% of anisoin, 0.02% benzylvanillin, 0.02% methylvanillin, 0.02% syringic acid, 0.02% cinnamic acid, 0.02% vanillic acid, veratric acid, 0.02% veratryl alcohol, 0.02% alkali lignin, 0.02% xylan, and 0.02% cellobiose as the sole sources of carbon. Starter culture was prepared by transferring strain 3k colonies to a flask containing 100 ml of 1/5 strength LB broth and grown for 24 hours at 30°C. Aliquots of 0.1 ml of appropriately diluted (10⁶-fold dilution) culture were used as the inoculum. Flasks were incubated static in the dark at 30 °C. Culture (1 ml) was withdrawn periodically from each flask and degradation of lignin compounds was determined by plating the appropriately diluted culture onto LB plates and colony-forming unit (CFU) was determined.

RESULTS AND DISCUSSION

One of the major environmental problems today is the contamination of soil and water by pollutants from petrochemical industries. Bioremediation is the promising technology for the remediation of contaminated sites since it is cost-effective and will lead to complete destruction of pollutants. Many indigenous microorganisms in water and soil are capable of degrading hydrocarbon contaminants. The primary goal of this study was to determine natural bioremediation potential of soil organisms. We intentionally contaminated garden soil with kerosene and attempted to isolate kerosene degrading organisms. Among the isolates, we chose Alcaligenes sp strain 3k for further study as this organism grew well on kerosene. Figure 1 shows the growth of strain 3k on hexadecane. The bacterium grew well on hexadecane as the sole carbon and energy source. Growth of the organisms increased from initial 67, 000 cfu to 30,500,000,000 cfu in 24 days suggesting an increase in population by almost 6-log unit. These data show that strain 3k grows well on hexadecane. This is important because hexadecane is present in the aliphatic fraction of crude oil and is one of the major components of diesel (Chénier et al., 2003). In addition, alkanes, especially longer-chain alkanes like hexadecane are non-polar molecules with very low chemical activity and water solubility, (Rojo 2009) hence; their degradation by microbes constitutes an important aspect of reclamation of polluted soil for agricultural purposes. Strain 3k showed growth on cyclohexane but not as much as it did on hexadecane (Figure 2). Interestingly, strain 3k did not utilize hexane as the carbon source (Table 1). These findings correlate with that of Zampolli et al. (2014), where Rhodococcus opacus R7 was unable to degrade hexane and octane. It was however able to degrade decane, dodecane, hexadecane, eicosane, tetracosane and hexatriacontane.

Figure 3 depicts the utilization of phenol as the sole carbon source by strain 3k. The organism degraded almost 80% of the initially added phenol within 7 days. Many researchers had previously reported Biodegradation of phenol. Bai et al. (2007) reported that Alcaligenes faeacalis could utilize 100 mg L⁻¹ phenol in just 10 hours as the sole source of carbon. Jiang et al. (2007) isolated A. faeacalis from acclimated activated sludge and it was shown to degrade high concentration of phenol (1600 mg L⁻¹) in 76 h. Agarry et al. (2008) reported that Pseudomonas aeruginosa NCIB 950 and Pseudomonas fluorescens NCIB 3756 were able to degrade phenol. Chakraborty et al. (2010) had reported that one of three local bacteria detected in coke processing water was able to degrade phenol. Zhenghui et al. (2016) reported the biodegradation of phenol by Acinetobacter calcoaceticus. They reported that the strain removed 91.6% of the initial 800 mgL⁻¹ phenol within 2 days. Environmental fate of phenol is important as this compound is toxic, recalcitrant, bioaccumulate in organisms and negatively affects aquatic biota (Annachatre & Gheewala, 1996).

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Strain 3k degraded > 90% of the added benzoate within 2 days of incubation (Figure 4). Benzoate has been widely used as a model compound for the study of the bacterial catabolism of aromatic compounds. Benzoate (or benzoyl-CoA) is the most common intermediate in anaerobic metabolism of aromatic compounds and recently, benzoate has been reported to be an intermediate of anaerobic benzene biodegradation (Caldwell & Suflita, 2000). *Pseudomonas* and *A. eutrophus* have been reported to degrade benzoate via the ortho cleavage pathway (Ampe *et al.* 1997; Feist & Hegeman, 1969) but other studies have shown the possibility of both meta and ortho pathways (Nakazawa & Yokota, 1973). In some organisms, benzoate is reportedly converted to catechol using chromosomally encoded enzymes. Catechol is then further degraded to trichloroacetic acid cycle intermediates by an ortho ring cleavage pathway (Harwood & Parales, 1996).

Strain 3k rapidly utilized lignin monomers and dimers as well as sugar polymers (Table 2). The ability to utilize lignocellulosic compounds is important for overall carbon cycling in soil (Datta *et al.* 2017) and for delignification of plant biomass for rapid saccharification of plant polysaccharides for efficient fermentation of sugars to biofuels and other chemicals (Brown & Chang 2014; Mosier *et al.* 2005). The observation that strain 3k with the ability to degrade aromatic compounds is also able to degrade lignin provides a possible link between aromatic degradation and lignin degradation, which is logical, given that lignin is the ultimate source for much of the aromatic material found in soil.

![Fig. 1. Degradation of hexadecane by Alcaligenes sp. strain 3k. Data are the mean of triplicate plate counts, and bars indicate ± standard deviation.](image1)

![Fig. 2. Degradation of cyclohexane by Alcaligenes sp. strain 3k. Data are the mean of triplicate plate counts, and bars indicate ± standard deviation.](image2)
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Fig. 3. Degradation of phenol by *Alcaligenes* sp. strain 3k. Data are the mean of triplicate tubes, and bars indicate ± standard deviation (Control = o).

![Graph showing phenol degradation over time](image)

Fig. 4. Degradation of benzoate by *Alcaligenes* sp. strain 3k. Data are the mean of triplicate tubes, and bars indicate ± standard deviation (Control = o).

![Graph showing benzoate degradation over time](image)

Table 1. Degradation of aliphatic and aromatic hydrocarbons by *Alcaligenes* sp. strain 3k

<table>
<thead>
<tr>
<th>Compound</th>
<th>Degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kerosene</td>
<td>++</td>
</tr>
<tr>
<td>Benzene</td>
<td>-</td>
</tr>
<tr>
<td>Toluene</td>
<td>-</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>-</td>
</tr>
<tr>
<td>Xylenes</td>
<td>-</td>
</tr>
<tr>
<td>Phenol</td>
<td>++</td>
</tr>
<tr>
<td>Benzoate</td>
<td>++</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>+</td>
</tr>
<tr>
<td>Hexane</td>
<td>-</td>
</tr>
<tr>
<td>Hexadecane</td>
<td>++</td>
</tr>
</tbody>
</table>

1. *Alcaligenes* sp. strain 3k was grown in MSM supplemented with a petroleum compound as the sole source of carbon. ++ Best growth, + poor growth, and - no growth.
Table 2. Degradation of lignocellulose compounds by *Alcaligenes* sp. strain 3k

<table>
<thead>
<tr>
<th>Lignocellulose compound</th>
<th>2 weeks</th>
<th>4 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vanillic acid</td>
<td>3.99E+05</td>
<td>7.00E+07</td>
</tr>
<tr>
<td>Veratric acid</td>
<td>6.00E+04</td>
<td>1.01E+07</td>
</tr>
<tr>
<td>Methylvanillin</td>
<td>2.00E+05</td>
<td>2.00E+05</td>
</tr>
<tr>
<td>Syringic</td>
<td>1.55E+05</td>
<td>2.00E+05</td>
</tr>
<tr>
<td>Veratryl alcohol</td>
<td>3.86E+05</td>
<td>5.10E+06</td>
</tr>
<tr>
<td>Cinnamic acid</td>
<td>5.70E+04</td>
<td>5.00E+07</td>
</tr>
<tr>
<td>Anisoin</td>
<td>2.00E+06</td>
<td>4.00E+05</td>
</tr>
<tr>
<td>O-Benzylvanillin</td>
<td>9.61E+05</td>
<td>1.01E+07</td>
</tr>
<tr>
<td>Alkali lignin</td>
<td>2.50E+05</td>
<td>2.00E+05</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>5.00E+05</td>
<td>5.45E+06</td>
</tr>
<tr>
<td>Xylan</td>
<td>5.00E+05</td>
<td>1.15E+07</td>
</tr>
</tbody>
</table>

1. All flasks were inoculated initially 8.62E+04 ± 1.03E+04.
2. Each flask was amended with 0.02% lignocellulosic compound as the sole source of carbon. Flasks were incubated at 30°C in the dark.

**CONCLUSION**

This study shows that strain 3k is capable of degrading both aliphatic and aromatic compounds and not BTEX compounds. Previous studies, however, have shown *Alcaligenes* ability to degrade BTEX compounds (Plaza et al. 2007; Yeom & Yoo, 2002) as well compounds present in produced water (Okoro & Amund, 2010; Igwo-Ezikpe et al. 2009). The reason that strain 3k lacks the ability to degrade BTEX compounds is not known. Overall, *Alcaligenes* have the ability to degrade common toxic pollutants found in the environment, thus can play important role in the reclamation of contaminated agricultural soil and aquatic environments. *Alcaligenes* sp. strain 3k is expected to degrade hydrocarbons and lignin-like compounds on the field just as it has been demonstrated in this laboratory scale experiment.

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