**RESEARCH ARTICLE**

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# Occurrence of hydrocarbon-degrading nitrogen-fixing bacteria in the rhizosphere of *Paspalum vaginatum* Sw.

**ABSTRACT**

This study investigated the influence of nitrogen-fixing hydrocarbon-degrading bacteria from the rhizosphere of plants in the biodegradation of crude oil in the polluted soil. Several nitrogen-fixing and crude oil utilizing bacteria were isolated from the rhizosphere of the turf grass *Paspalum vaginatum* Sw. using crude oil as carbon and energy source. The isolates showed between 98% - 99% 16S rRNA gene similarity with species of *Alcaligenes*, *Brevundimonas* and *Klebsiella*. Three of the isolates, *Brevundimonas diminuta* C4B, *Alcaligenes faecalis* B5 and *Alcaligenes faecalis* D4A with the ability to grow well in crude oil as carbon and energy source were selected for further studies. *Alcaligenes faecalis* B5 had the highest growth rate constant of 0.038 $\mu^{-1}$ with a mean generation time of 18.46 h. Analysis of culture extracts by gas chromatography with flame ionization detector showed that *Alcaligenes faecalis* B5 degraded 90.5% of crude oil in 24 days, while *Alcaligenes faecalis* D4A and *Brevundimonas diminuta* C4B biodegraded 84.6% and 82.1% of the oil respectively. Only *Alcaligenes faecalis* C1B, *Klebsiella varicola* C3B and *Klebsiella varicola* C3BB had the *nifH* gene; and the genes responsible for nitrogen-fixing abilities of all the isolates were not plasmid-borne. Confirmation of nitrogen-fixing ability was determined by the acetylene reduction assay (ARA). *Alcaligenes faecalis* B5 produced the highest amount of ethylene (15.18 nmol) after 24 h of incubation, while *Alcaligenes faecalis* D4A and *Brevundimonas diminuta* C4B produced 12.10 nmol and 9.60 nmol of ethylene respectively. Nitrogen-fixing hydrocarbon utilizers are therefore potential seeds in the bioaugmentation of nitrogen-limited hydrocarbon-polluted environments.

**Key words:** Nitrogen fixers, biodegradation, acetylene reduction, ethylene, rhizodegradation, rhizosphere, crude oil

## Introduction

Petroleum is considered essential for the industrialized society. Nigeria's oil industry, one of the leading producers in the world, has generated myriads of pollutants in the form of gaseous emissions, oil spills, effluents and solid wastes (Chikere et al., 2012). The Niger Delta area of Nigeria has been the most affected as a result of the upsurge in oil exploration and exploitation activities mostly undertaken by multinational companies. Degradation of petroleum has been known for over 50 years, whilst hydrocarbon-oxidizing bacteria have mostly been isolated from surface areas such as soils, petroleum storage tanks and oil spill sites (Huy et al., 1999; Das & Chandran, 2011). The use of pure bacterial strains for the biodegradation of individual hydrocarbon compounds has been studied extensively, and metabolic pathways have been described (Kebria et al., 2009). The biodegradation of crude oil in the environment occurs at varying rates, depending on numerous physical and biogeochemical perturbations imposed on these ecosystems (Enock, 2002). These perturbations include such factors as temperature, pH, oxygen and nutrient availability (Sihag et al., 2014). Acuna et al. (2012) reported that nitrogen deficiency during biodegradation produced a decrease in the mineralization, hydrocarbon elimination and biomass, hence the need to augment bioremediation sites with artificial sources of nitrogen. Different types of grass-associated diazotrophic endophytes that belong to α, β and γ subclasses of the proteobacteria have been identified (Triplett, 1996; Fernández et al., 2014).

*Paspalum vaginatum* Sw. also known as seashore Paspalum is a warm season, salt-tolerant, prostrate-growing turfgrass that can be found in tropical, subtropical and warm temperate regions of the world (Duncan, 1997). It is highly tolerant of various environmental stresses. Seashore Paspalum can form a higher quality turf in reduced light conditions, in soils ranging in pH from 3.6 to 10.2, in

waterlogged soils, and with fewer applications of nitrogen fertilizer (Brosnan & Deputy 2008). It tolerates salinity regimes ranging from freshwater to hypersalinity conditions of 50 ppt (Lonard et al., 2015). Bamidele & Igiri (2011) studied the growth of *Pasplum vaginatum* growing on crude oil contaminated soil. Their report suggests the potential use of *P. vaginatum* in oil-contaminated sites, either for protecting sediment from erosion, enhancing habitat restoration or accelerating oil degradation by generating a more aerobic soil environment.

Limiting nutrient such as nitrogen poses a challenge during remediation of oil-contaminated soils, leading to an increase in the cost of the process when nitrogen in form of fertilizer is used to stimulate the soil (Akpe et al., 2015a). Akpe et al. (2015b) reported enhancement in the biodegradation rate of crude oil polluted soil amended with cassava peels (an organic waste) that provided up to 2.37% nitrogen. Thus, one of the pressing research needs for biodegradation based oil spill remediation strategies is determining and evaluating the biotic and abiotic factors that influence the fate of the spilled oil and devising ways to accelerate the biodegradation rate. This study was undertaken to obtain a better understanding of the influence of nitrogen-fixing bacteria from the rhizosphere of plants in the biodegradation of crude oil in polluted environments.

## Materials and Methods

### *Sample collection*

Rhizosphere soil samples were collected from the rhizosphere of *Paspalum vaginatum* Sw. from five different spots at the Botanical Garden (BG) and Distance Learning Institute (DLI) Road, University of Lagos. Samples were collected into sterile bottles and transported to the laboratory. Physico-chemical and microbiological analysis commenced immediately upon arrival at the laboratories.

### *Source of crude oil*

Bonny Light crude oil sample used for the degradation study was obtained from a flow station of the Shell Petroleum Development Company of Nigeria, 12 km from Port-Harcourt Terminal.

### *Isolation of crude oil-utilizers*

Crude oil-utilizing nitrogen-fixing bacteria were isolated on Nitrogen-Free Minimal Salts Medium (NFMSM). The medium contained (g $L^{-1}$) $K_2HPO_4$ (1.0), $MgSO_4.7H_2O$ (0.2), $CaCO_3$ (1.0), NaCl (0.2), $Na_2MoO_4.2H_2O$ (0.005), $FeSO_4.7H_2O$ (0.1), Agar (15.0), and 1000 ml of distilled water, pH 7.2. Sterile crude oil (1%) served as the sole carbon and energy source and was made available to the culture through vapor phase transfer (Amund & Akangbou, 1993). The inoculated plates were incubated aerobically at room temperature for 5-7 days.

### *Preparation of starter cultures*

Crude oil-utilizing nitrogen-fixing bacteria grown for 24 h were harvested from the surface of Luria Bertani Agar (LBA) and re-suspended in 10 ml sodium phosphate buffer (50 mM, pH 7.2) in a test tube. Centrifugation was carried out at 10,000×g for 10 min. The supernatant was discarded while the pelleted cells were re-suspended in the same buffer and the washing repeated for two more times at the same condition. The pelleted cells were finally re-suspended in sterile NFMSM and used for crude oil degradation studies (Iiori & Amund, 2000).

### *Biodegradation studies*

The degradation of crude oil as carbon and energy source by the isolates and not as opportunistic utilizers on the agar used as solidifying medium was done by inoculating each isolate in separate cotton wool-plugged 250 ml Erlenmeyer flasks containing sterile liquid NFMSM and crude oil. The medium and crude oil were separately autoclaved at 121oC for 15 min. One milliliter of crude oil was added to 99 ml of NFMSM after which each isolate was inoculated into separate flasks appropriately. Control flasks containing 100 ml of a medium of test crude oil without the isolates and with autoclaved isolates were also prepared. Flasks were incubated at room temperature for 14 days with constant agitation. Turbidity was measured by determining optical density (OD 520nm) using a spectrophotometer (Jenway 6305, Essex, UK). Isolates with the highest OD were selected for further study. The growth-time experiment of the selected isolates was monitored for 24 days and the residual crude oil content before and after biodegradation was analyzed by gas chromatography.

### *Emulsification index (E24)*

The emulsifying capacity was evaluated by an emulsification index (E24). The E24 of culture samples were determined by centrifuging 2 ml of spent medium at 3,000×g for 15 min to separate the cells. The emulsification stability of the isolates was determined by adding 2 ml of crude oil to the test tubes containing the spent media. The tubes were vortexed at high speed for 2 min and allowed to stand for 24 h. The emulsification index (E24) was defined as the height of the emulsion divided by the total height of supernatant with added oil multiplied by 100 (Abasi & Amiri, 2009).

### *Characterization and identification of isolates*

The isolates were identified based on a conventional biochemical test using the Analytical Profile Index (API) RAPID ID 32E kit. The kit was prepared according to the manufacturer's specification. Further molecular identification was done by analysis of the 16S rRNA gene sequence.

***Molecular identification***

The total genomic DNA was extracted using the MoBio soil DNA isolation kit as described by the manufacturer (The Mo Bio Laboratories, INC). The Polymerase chain reaction (PCR) amplification of the 16S rRNA gene was carried out using the primer set U1- 5'-CAG CMG CCG CGG TAA TWC-3' and U2 5'-CCG TCA ATT CMT TTR AGT TT-3'. The reaction was carried out in 25 µl reaction mixture containing 1X PCR buffer (Solis Biodyne, Tartu, Estonia), 1.5 mM Magnesium chloride, 0.2 mM of each dNTP, 1 U Taq DNA Polymerase (Solis Biodyne, Tartu, Estonia), 20 pMol of each primer and sterile double distilled water was used to make up the reaction mixture. The PCR was carried out in an Eppendorf Nexus thermal cycler with the following cycling parameters; an initial denaturation step at 95°C for 5 min, followed by 30 consecutive cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 1 min and 72°C for 1 min. After this, a final extension at 72°C for 10 min was carried out. The PCR products were separated on a 1.5% agarose gel. Electrophoresis was done at 80V for 2 h, and the gel was viewed under UV light after ethidium bromide staining.

The PCR products were purified and sequenced by GATC Biotech AG, European Genome and Diagnostics Centre, Konstanz, Germany. The 16S rRNA gene fragments were identified by probing against GenBank with the BLAST algorithm.

***Nucleotide sequence accession numbers***

The sequence data for the bacterial isolates have been deposited at GenBank under accession numbers KF929386 to KF929395. Phylogenetic tree with retrieved sequences from NCBI database was constructed using the neighbor-joining algorithm with the program MEGA.5 (Tamura et al., 2011).

***Plasmid profiling***

Plasmid extraction was carried out using the STET method. Overnight cultures were harvested into 300 µl of STET buffer (8% Sucrose, 5% Triton X-100, 50 mM EDTA, and 50 mM Tris-HCL pH 8.0). Cells were allowed to homogenize in STET buffer for 10 min in a thermomixer. After homogenization, 15 µl of 10 mg ml-1 lysozyme in STET buffer was added to the mixture, mixed and incubated on ice for 5 min. The samples were boiled at 99°C for 1 min. After boiling, the samples were centrifuged for 10 min at 10,000 rpm, and the supernatant transferred into another tube.

Ice cold ethanol (500 µl) was added to the supernatant, and the samples were incubated for 20 min on ice. This was followed by centrifuging at 10,000 rpm for 15 min. The supernatant was discarded, and pellets were washed twice in 70% ethanol. The pellets were allowed to dry at room temperature and then dissolved in 50 µl of TE buffer. The dissolved plasmids were separated using a 0.8% agarose gel and viewed under UV light after ethidium bromide staining.

***Detection of nifH gene***

The presence of *nifH* gene was determined as described by Chowdhury et al. (2009). A pair of universal degenerate primers Zehr-*nifHf* (5'-TGYGAYCCNAARGCNGA-3') and Zehr-*nifHr* (5'-ADNGCCATCATYTCNCC-3'), were used to amplify a 360 bp fragment of the *nifH* gene (Tan et al., 2003).

***Acetylene reduction assay***

Isolates were inoculated into a freshly prepared nitrogen-free medium in serum bottles and incubated. Cotton wool plugs from culture bottles were replaced with rubber stoppers, and 1 ml of air was removed from the bottles with a syringe. One millilitre of acetylene was injected into the bottles and incubated for 24 h. One millilitre samples were analyzed for the reduction of acetylene to ethylene by chromatography (Holguin et al., 1992)

***Generation of acetylene gas***

Acetylene gas was produced by reacting rocks of calcium carbide with water ($CaC_2 + 2H_2O \rightarrow C_2H_2 + Ca(OH)_2$) in a cylinder with a vent. A Tygon tube was connected to the vent and linked to a gas collection bag (Dilworth, 1966).

***Gas chromatography (GC) analysis***

A Hewlett Packard (HP) 5890 series II with a flame ionization detector (FID) was used. Instrument operating conditions were as follows: an OV-3 glass column pack with internal diameter of 5.3 m and length of 30 m packed with porapak N, 60/100, a column temperature of 200$^{o}$C, an injector temperature of 60$^{o}$C, a detector temperature of 280$^{o}$C, N2 carrier gas and H2 at a flow rate of 22 ml min$^{-1}$ and temperature/ramping rate of 5$^{o}$C min$^{-1}$. The injection volume was 1 µl.

***Statistical analysis***

Statistical analyses of the mean and analysis of variance at $P < 0.05$ were carried out using the GraphPad Prism version 5.0 computer software programme (GraphPad Software, San Diego, CA. USA).

## Results

***Characterization and identification of nitrogen-fixing, crude oil utilizing isolates***

The isolates were biochemically identified as species of *Pseudomonas* and *Klebsiella*. Molecular characterization based on 16S rRNA partial sequence showed that the strains shared between 98%-99% gene similarity with *Alcaligenes*, *Brevundimonas* and *Klebsiella*. They were identified as *Alcaligenes faecalis* strain A5B1, *Alcaligenes faecalis* strain

A5B2, *Alcaligenes faecalis* strain B5, *Alcaligenes faecalis* strain C1B, *Brevundimonas diminuta* strain C4B, *Brevundimonas diminuta* strain C3B, *Klebsiella variicola* strain C3B, *Klebsiella variicola* strain C3BB, *Alcaligenes faecalis* strain D4A and *Alcaligenes faecalis* strain D3. The accession numbers (KF929386 to KF929395) of the nucleotide sequences of the isolates deposited at NCBI GenBank database is shown in Table 1. *NifH* gene was detected only in *Alcaligenes faecalis* strain C1B, *Klebsiella variicola* strain C3B and *Klebsiella variicola* strain C3BB. None of the isolates harboured plasmids. The phylogenetic tree of the isolates and related bacterial strains retrieved from NCBI database is shown in Figure 1. The *Alcaligenes* and *Brevundimonas* spp share a similar evolutionary relationship with *Pseudomonas stutzeri* (AF143245). It can also be inferred from the tree, that there is comparable sensitivity in identification of the isolates by both the conventional and molecular methods.

***Biodegradation studies***

*Alcaligenes faecalis* strain B5, *Brevundimonas diminuta* strain C4B and *Alcaligenes faecalis* strain D4A were selected for further studies, based on their abilities to grow well in crude oil as carbon and energy source. The growth dynamics of the isolates in NFMSM with crude oil as the sole carbon and energy source is displayed in Figure 2 and Table 2. All isolates had a lag phase of about 2 days. *Alcaligenes faecalis* B5 had a faster exponential growth rate with an initial population of 5.4×103 cfu $ml^{-1}$ and a final population density of 2.01×108 cfu $ml^{-1}$ in 24 days (Figure 2). Its growth rate constant (μ) was 0.038 (Table 2), and it had a higher emulsification index of 28.7% (Figure 2). *Brevundimonas diminuta* C4B had a growth rate constant of 0.025 with a mean generation time (g) of 27.69 h and entered stationary phase around day 18 having a population of about 8.2×109 cfu $ml^{-1}$. The emulsification activity (E24) of *Brevundimonas diminuta* C4B was 23.95% at day 24 while *Alcaligenes faecalis* D4A had percentage emulsification index of 30.56. The statistical analyses of variance at $P < 0.05$ showed no differences in the degradation, growth rate constant and emulsification capacity of crude oil by the isolates (Figure 2 and Table 2).

***Gas Chromatographic profiles***

The gas chromatography analysis of culture extracts from the biodegradation set-up after 24 days showed a reduction in the amount of residual crude oil present in the media (Table 3). The amount of crude oil present in the liquid medium at the start of the experiment was 2714.87 mg $L^{-1}$. The amount of residual crude oil present at day 24 for *Alcaligenes faecalis* B5, *Brevundimonas diminuta* C4B and *Alcaligenes faecalis* D4A were 258.07 mg $L^{-1}$, 487.20 mg $L^{-1}$ and 418.56 mg $L^{-1}$ respectively. *Alcaligenes faecalis* B5 had the highest degradation rate of 90.5% followed by *Alcaligenes faecalis* D4A (84.6%) and *Brevundimonas diminuta* C4B (82.1%).

***Acetylene reduction assay***

Acetylene reduction assay carried out on *Alcaligenes faecalis* B5, *Brevundimonas diminuta* C4B and *Alcaligenes faecalis* D4A after 24 h of incubation confirmed that they were nitrogen fixers (Table 4). *Alcaligenes faecalis* B5 produced the highest volume of ethylene (15.18 nmol) from 1 ml of acetylene gas introduced into 4 ml of culture after 24 h incubation period. This was closely followed by *Alcaligenes faecalis* D4A which produced 12.10 nmol and *Brevundimonas diminuta* C4B produced the lowest concentration of 9.60 nmol ethylene. A5B2, *Alcaligenes faecalis* strain B5, *Alcaligenes faecalis* strain C1B, *Brevundimonas diminuta* strain C4B, *Brevundimonas diminuta* strain C3B, *Klebsiella variicola* strain C3B, *Klebsiella variicola* strain C3BB, *Alcaligenes faecalis* strain D4A and *Alcaligenes faecalis* strain D3. The accession numbers (KF929386 to KF929395) of the nucleotide sequences of the isolates deposited at NCBI GenBank database is shown in Table 1. *NifH* gene was detected only in *Alcaligenes faecalis* strain C1B, *Klebsiella variicola* strain C3B and *Klebsiella variicola* strain C3BB. None of the isolates harboured plasmids. The phylogenetic tree of the isolates and related bacterial strains retrieved from NCBI database is shown in Figure 1. The *Alcaligenes* and *Brevundimonas* spp share a similar evolutionary relationship with *Pseudomonas stutzeri* (AF143245). It can also be inferred from the tree, that there is comparable sensitivity in identification of the isolates by both the conventional and molecular methods.

**Table 1.** *Molecular Identification of isolates from the rhizosphere of Paspalum vaginatum*.

| Organism Code | Phenotypic characterization | 16S rRNA Identification | % Similarity with closest | GenBank Accession Number | *nifH* | Plasmid |
|---|---|---|---|---|---|---|
| D4A | *Pseudomonas* spp | *Alcaligenes faecalis* | 99% | KF929386 | - | - |
| D3B | *Pseudomonas* spp | *Alcaligenes faecalis* | 99% | KF929387 | - | - |
| C5A | *Pseudomonas* spp | *Alcaligenes faecalis* | 99% | KF929388 | - | - |
| B5 | *Pseudomonas* spp | *Alcaligenes faecalis* | 99% | KF929389 | - | - |
| C1B | *Pseudomonas* spp | *Alcaligenes faecalis* | 99% | KF929390 | + | - |
| A5B2 | *Pseudomonas* spp | *Alcaligenes faecalis* | 98% | KF929391 | - | - |
| CAB | *Pseudomonas* spp | *Brevundimonas* | 99% | KF929392 | - | - |
| A5B1 | *Pseudomonas* spp | *Brevundimonas* | 99% | KF929393 | - | - |
| C3B | *Klebsiella Pneumponia* | *Klebsiella variicola* | 99% | KF929394 | + | - |
| C3BB | *Klebsiella Pneumponia* | *Klebsiella variicola* | 99% | KF929395 | + | - |

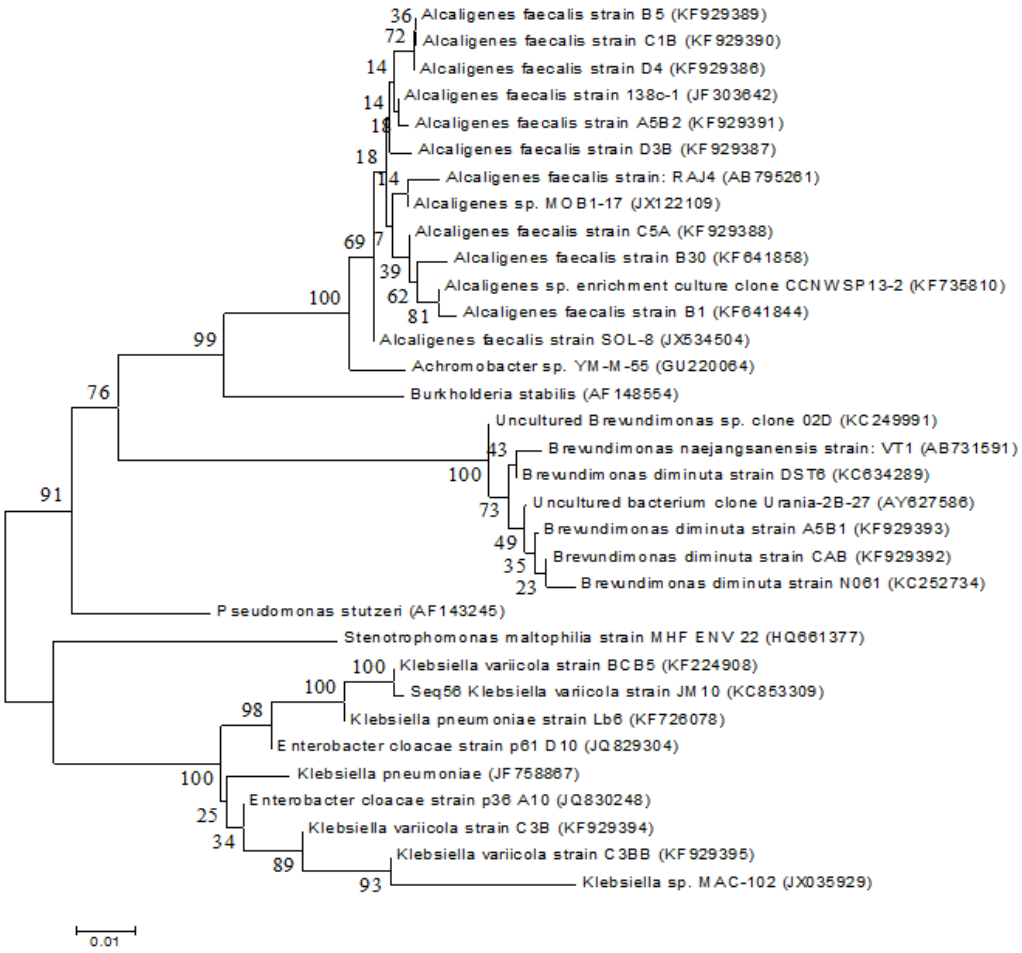


**Figure 1.** *Phylogenetic tree from neighbor-joining analysis of 16S rRNA partial sequence analysis of nitrogen-fixing hydrocarbon-degrading rhizosphere bacteria and related species retrieved from NCBI GenBank. Accession number of each bacterium is shown after the species name.*

**Table 2.** *Growth kinetics of isolates in liquid media.*

| Isolates | *Alcaligenes faecalis* strain B5 | *Brevundimonas diminuta* strain C4B | *Alcaligenes faecalis* strain D4A |
|---|---|---|---|
| Mean generation time (g) h | 18.46 | 27.69 | 22.92 |
| Growth rate constant ($\mu$ $h^{-1}$) | 0.038 | 0.025 | 0.030 |

**Table 3.** *Residual concentrations of crude oil in liquid cultures during biodegradation studies.*

| *Sample* | *Days* | *Residual Crude Oil (mg $L^{-1}$)* | *% Biodegradation* |
|---|---|---|---|
| Control | 0 | 2714.87 | - |
| Control | 24 | 1944.13 | 28.3 |
| *Alcaligenes faecalis B5* | 24 | 258.07 | 90.5 |
| *Brevundimonas diminuta C4B* | 24 | 487.20 | 82.1 |
| *Alcaligenes faecalis D4A* | 24 | 418.56 | 84.6 |

**Table 4.** *Acetylene reduction assay of nitrogen-fixing crude oil utilizers.*

| *Sample* | *Amount of Ethylene (nmol) 24 $h^{-1}$* | *Amount of Ethylene Produced Per cell (nmol) 24 $h^{-1}$* |
|---|---|---|
| Control | - | - |
| *Alcaligenes faecalis B5* | 15.18 | 0.0434 |
| *Brevundimonas diminuta C4B* | 9.60 | 0.0347 |
| *Alcaligenes faecalis D4A* | 12.10 | 0.0381 |

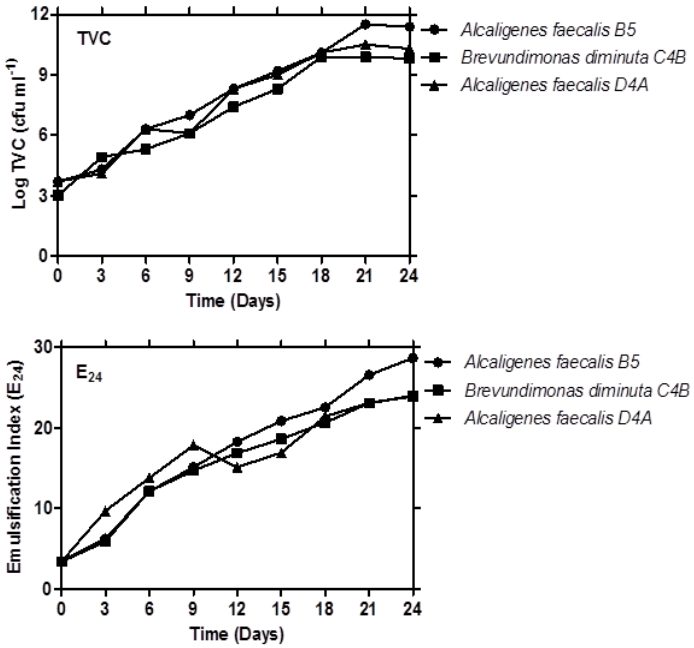


**Figure 2.** *The growth profile and emulsification capacity of isolates from the rhizosphere of Paspalum vaginatum in crude oil (Log TVC- Logarithm of Total viable count; cfu $ml^{-1}$- colony forming units per millilitre).*

## Discussion

The rhizosphere soil of the plant *Paspalum vaginatum* harbored several species of the nitrogen-fixing hydrocarbon-utilizing bacteria of the genera *Alcaligenes*, *Brevundimonas*, and *Klebsiella*. These genera of bacteria have been reported in other studies (Tumaikina et al., 2008; Igwo-Ezikpe et al., 2009; Rodrigues et al., 2009; Rosenblueth et al., 2004) to have hydrocarbon degrading potentials and nitrogen-fixing ability. Döbereiner & Day (1976) and Vose (1983) also described species of other nitrogen fixing bacteria such as *Azotobacter*, *Azospirillum* and *Herbaspirillum*, associated with the rhizosphere of several tropical grasses such as *Paspalum* and *Digitaria* (Dalton et al., 2004).

In our study, *Alcaligenes* and *Brevundimonas* were all identified as *Pseudomonas* spp by conventional analysis but the molecular analysis picked up the inherent differences in the genetic make-up of the isolates and placed them into two groups, while the neighbor-joining analysis showed their evolutionary relatedness to *Pseudomonas*. The inference is that these genera could have originated from a common ancestor. This observation supports other studies that have demonstrated the increased sensitivity of molecular identification over conventional biochemical identification of bacteria (Heikens et al., 2005; Rhoads et al., 2012). Liu et al. (2012) reported the phylogenetic diversity of nitrogen-fixing bacteria obtained from the mangrove of rhizosphere soil analyzing the *nifH* gene. Their results revealed that most isolates belonged to the Gammaproteobacteria *Pseudomonas*, which supports our observation.

Nitrogen fixation is carried out by the nitrogenase enzyme whose multiple subunits are encoded by the genes nifH, nifD and nifK as reviewed by Rubio & Ludden (2002) (Gaby & Buckley, 2012). The *nifH* gene encoding the nitrogenase reductase subunit is the most sequenced and has become the biomarker most widely used in ecology and evolutionary studies of nitrogen-fixing bacteria (Raymond et al., 2004; Gaby & Buckley, 2012). The *nifH* primers used in this study was reported by Chowdhury et al. (2009) to have been widely and successfully used to amplify *nifH* gene from distantly related diazotrophs by Zehr & McReynolds (1989). However, the primer was unable to amplify the genes in some of the species from the genera *Alcaligenes* and *Brevundimonas*. The non-amplification of the *nifH* genes in the isolates does not depict absence of the nitrogenase reductase subunit as there are numerous universal *nifH* primers which vary in their phylogenetic bias and their ability to recover sequences from commonly sampled environments (Gaby & Buckley, 2012).

Plasmids were not detected in any of the bacteria studied, indicating a chromosomal location of hydrocarbon-utilizing genes in the organisms. This is in line with the results of Kehinde & Isaac (2017) that reported chromosomally mediated degradation of crude oil by bacteria of soil origin.

The increase in the emulsification capacity of the isolates with the corresponding increase in bacterial population density and the overall hydrocarbon degradation capacity showed that the bacterial isolates were responsible for oil degradation. Similar results have been reported by Mandri & Lin (2007), Obayori et al. (2009) and Omotayo et al. (2012). The loss of hydrocarbon components recorded in the control sample may be attributed to volatilization or photolysis, which occurred during the period of the study. According to Fingas (2011, 2013), evaporation is an integral process in oil spills and up to 45% of the crude oil, mainly in the form of volatile hydrocarbons (BTEX etc.), may evaporate in the first few days, depending on the oil thickness and temperature.

Degradation of hydrocarbons in the environment is influenced by the availability of nutrients such as nitrogen and phosphate (Leahy & Colwell, 1999). Thus, rhizodegradation of hydrocarbon polluted soils need not be augmented with other nitrogen sources such as fertilizers since rhizosphere bacteria are capable of fixing atmospheric nitrogen. The acetylene reduction activity obtained from the isolates were in line with the study of Lü & Huang (2010) whose bacteria isolates had acetylene reduction activity ranging from 0.048 nmol of ethylene to 29.54 nmol ethylene. The work of Holguin et al. (1992) also showed isolates with appreciable acetylene reduction activity after 24 hours incubation period.

This work has shown the occurrence of various strains of bacteria around the rhizosphere of *Paspalum vaginatum* capable of degrading hydrocarbon. Not only do they degrade hydrocarbon they also fix nitrogen, an important limiting nutrient for plants and soil fertility. It is therefore pertinent to recommend that plants that can withstand the pressure of hydrocarbon pollution and at the same time support the proliferation of nitrogen-fixing organisms should be planted in areas polluted or prone to hydrocarbon contamination, as this will enhance rhizoremediation of the polluted soils. In addition, the nitrogen-fixing hydrocarbon utilizing bacteria associated with the rhizosphere of these plants could serve as potential seeds for the bioaugmentation of nitrogen-limited oil-polluted environments.

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