Full Length Research Paper

Characterisation and evaluation of the efficiency of petroleum degrading bacteria isolated from soils around the oil exploration areas in western Uganda

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Contamination with petroleum and its products is an environmental threat in oil producing countries. Microbes have been used to clean up petroleum contaminated environments, which has been demonstrated to be an appropriate and more practical alternative compared to the mechanical and chemical techniques. In this study, crude oil degrading indigenous bacteria were isolated from soils around the oil exploration sites in Western Uganda and their efficiency of oil biodegradation and presence of the catabolic gene *xylE* in the isolates to relate with their biodegradation efficiency were determined. The organisms with oil degrading activity were screened by isolating them from crude oil supplemented mineral salts medium (MSM). The isolates were tentatively identified phenotypically and confirmed genotypically by 16S ribosomal ribonucleic acid (rRNA) gene sequencing. Hydrocarbon degradation in the culture fluids was analyzed by a gas chromatography mass spectrometer (GC/MS); and amplification of catabolic gene *xylE* by polymerase chain reaction (PCR) was done in order to relate with the degradation activity. Forty four indigenous oil degrading bacteria were isolated and categorized into eight groups based on their morphological and biochemical properties; and were identified as belonging to the genera; *Corynebacterium, Pseudomonas, Moraxella, Bacillus, Enterobacteriaceae, Nocardia, Serratia* and *Rhodococcus*. Eight isolates that exhibited a relatively higher biodegradation activity in the first five days of incubation were selected for a detailed analysis. Based on 16S rRNA gene sequence analyses, the eight selected isolates were identical to *Stenotrophomonas maltophilia, Burkholderia* sp., *Delftia tsuruhatensis, Pseudomonas aeruginosa, Acinetobacter* sp., *Curtobacterium* sp. and *Paenibacillus agaridevorans.* The selected isolates; Ngara-T1, Kig5-1, Kig5-T2, Kig3-T3, Kig3-T4, Kas2-T7, Kas2-T5 and Gat-3 degraded the total petroleum hydrocarbons (TPHs) up to: 91.9, 79.9, 89.2, 73.1, 94.2, 83.2, 77.0 and 67.2%, respectively, by the end of 21 days of incubation as compared to 29.7% degradation in the control experiment (without bacteria). The catabolic gene *xylE* was detected in two of the selected isolates, Ngara T-1 and Kig5-T2. It could be concluded that the oil degrading bacteria identified in this study showed diverse and varying capacities to degrade the crude oil; with some degrading up to 90% and can be exploited for cleaning up hydrocarbon contaminated sites in western Uganda.

Key words: Bacteria, bioremediation, degradation, oil, hydrocarbons, Uganda.

INTRODUCTION

The term petroleum is used as a common denotation for crude oil and natural gas, which are the hydrocarbons from which various oil and gas products are made (JICA and EEAA, 2008). Crude oil is a complex mixture of thousands of different chemical components, mainly organic compounds which usually make up about 95%. At the refinery, crude oil is separated into light and heavy fractions, which are then converted into various products, such as petrol, diesel oil, jet fuel and lubricating oils through processes such as catalytic cracking and fractional distillation (JICA and EEAA, 2008; Zhu et al., 2001).

During routine operations of extraction, transportation storage, refining and distribution, accidental spills of crude oil and its refined products occur on a frequent basis despite the counter measures put in place (Zhu et al., 2001). It is approximated that five million tons of crude oil and refined oil enter the environment each year and most of it is directly related to human activities including deliberate waste disposal (Malatova, 2005). The oil spill incident from British Petroleum in the Gulf Coast of Mexico from April to July, 2010, caused almost 600,000 tons of crude oil spilled along the Gulf Coast (Wang et al., 2011). South Africa which is situated on one of the world's major shipping routes, is constantly sub-jected to conta-mination of its coastal waters by oil spills that leak from passing traffic (Moldan and Dehrman, 1989). Also, the National Petroleum Corporation in Nigeria placed the quantity of petroleum jettisoned into the environment at 2,300 cubic meters of oil spilled in 300 separate incidents annually (Manby, 1999).

Petroleum contains polycyclic aromatic hydrocarbon (PAHs) compounds such as benzene, toluene, ethyl benzene, *xyle*nes and naphthalene, which are among the most toxic components to plants and animals (Atlas and Hazen, 1999; Liebeg and Cutright, 1999). The aromatic hydrocarbons are more resistant against biodegradation than aliphatic compounds and they often cause serious problems during bioremediation; hence, it is very important to minimize contamination of the environment with the petroleum constituents (Rajaei et al., 2013).

The mechanical strategies for containment of oil spills typically recover no more than 10-15% of the oil after a major spill (Zhu et al., 2001). Bioremediation has emerged as one of the most promising secondary treatment options for oil removal which is often used as a polishing step after conventional cleanup options have been applied (Bragg et al., 1994; Zhu et al., 2001).

Bioremediation is done by adding materials such as microbes and nutrients (phosphorus and nitrogen) to contaminated environments, such as oil spill sites, to cause an acceleration of the natural biodegradation processes (Zhu et al., 2001). Hydrocarbons are biode-graded using microbial organisms such as bacteria, protozoa and fungi, which degrade contaminants com-pletely to biomass, carbon-dioxide and water and utilize the resulting compounds as nutrients and energy sources for growth and reproduction (Frick et al., 1999). Several genes associated with bacteria have been evaluated for bioremediation processes. The catabolic gene *xylE* which codes for catechol 2,3-dioxygenase that cleaves the aromatic rings in PAHs is one of the genes identified in hydrocarbon degrading bacteria that are evaluated for use in hydrocarbon contaminated sites for bioremediation processes (Malkawi et al., 2009; Rajaei et al., 2013).

Bioremediation has a proven track record and it has been used successfully to clean up spills of oils and other hydrocarbons for more than 20 years (Frick et al., 1999). Compared to physicochemical methods, bioremediation offers an effective technology for the treatment of oil pollution because the majority of molecules in the crude oil and refined products are biodegradable and oil-degrading microorganisms are ubiquitous (Aislabie et al., 1998). According to Mittal and Singh (2009), the most direct measure of bioremediation efficacy is by monitoring of hydrocarbon disappearance rates. In addition to measuring Total petroleum hydrocarbons (TPH) in samples, the gas chromatography/flame ionization detector (GC-FID) chromatograms provide a distribution pattern of petroleum hydrocarbons fingerprints of the major oil components and information on the biodegradation extent of the spilled oil.

Since oil exploration and production is a new activity in Uganda, little or no research has been done in the country to investigate the presence of oil degrading bacteria in soils. Therefore, there is need to explore and prepare the techniques for thorough cleanup of oil spills as the latter cannot be predicted when and where they would occur despite any measures put in place. Hence, the aim of this study was to explore the possibility of bioremediation by isolating bacteria that occur in the soils in oil exploration and production sites in Western Uganda, and to determine their oil biodegradation capabilities.

METHODOLOGY

Study sites

Samples of soil for this study were collected from four petroleum exploration fields; Ngara, Kigogole 5, Kigogole 3 and Kasamene 2 all found in Buliisa district in western Uganda. Soil samples contaminated with oil were also collected from Gatsby motor garage located in Makerere University, Kampala, Uganda. Samples of

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petroleum, earlier collected from Ngara-1 exploration site, were obtained from the Petroleum Exploration and Production Department (PEPD) located at Entebbe, Uganda.

Sample collection

Subsurface soils (500 g) contaminated with petroleum were picked from the study sites, labeled and transported in pre-sterilized polythene bags in a cooler box. The samples (in 20% glycerol as a cryopreservative) were stored at -20°C in the microbiology laboratory at the College of Veterinary Medicine, Makerere University.

Preparation of media

The enrichment medium (MSM) consisted of the following salts: $NaNO_3$ (7 g/l); K_2HPO_4 (1 g/l); KH_2PO_4 (0.5 g/l); KCl (0.1 g/l); $MgSO_4$-$7H_2O$ (0.5 g/l); $CaCl_2$ (0.01 g/l) and $FeSO_4$-$7H_2O$ (0.01 g/l). The medium was supplemented with trace elements solution (0.05 ml) composed of the following salts: H_3BO_3 (0.25 g/l); $CuSO_4$-$5H_2O$ (0.5 g/l); $MnSO_4$-H_2O (0.5 g/l); $MoNa_2O4$-H_2O (0.06 g/l) and $ZnSO_4$-$7H_2O$ (0.7 g/l) (Kebria et al., 2009). The petroleum agar was prepared by adding 10 ml of crude oil and 20 g of bacteriological agar in a liter of prepared mineral salts medium.

Isolation of oil degrading bacteria

The isolation of petroleum degrading bacteria was done according to the method described by Mittal and Singh (2009). Briefly, 10 g of soil sample were suspended in 30 ml of distilled water in a falcon tube and mixed thoroughly for two minutes by vortexing. The suspension was allowed to settle down for five minutes and 5 ml of supernatant were inoculated in 100 ml of mineral salt medium (MSM) supplemented with crude oil (1%) as the sole source of carbon and energy. The flasks were incubated at room temperature (25 ± 2.0°C) on a rotary shaker at 150 rpm for 48 h. Successive sub-culturing was done by transferring 5 ml of MSM broth culture onto a fresh mineral salt medium supplemented with crude oil (1%). The sub-culturing was done to ensure that only oil-tolerant and -degrading bacteria would be isolated. After three sub-culturing steps, the oil-degrading bacteria were isolated by spreading out 1 ml of the broth culture onto petroleum agar plates. The inoculated plates were incubated aerobically at room temperature in an incubator for four days.

Phenotypic identification of the bacterial isolates

The bacterial colonies that grew on petroleum agar plates were selected based on the colony shape, colour, size and elevation. The selected colonies were sub-cultured on nutrient agar for pure culture preparation and identification. The bacterial isolates were identified on the basis of their colonial and cellular morphology; and biochemical characteristics. The colony colour, margin, form and elevation of the isolates were noted. Gram staining and biochemical tests including; catalase, oxidase, glucose and maltose fermentation, indole, urease, citrate and haemolysis tests were also performed to identify the pure colonies. The pure colonies were preserved in glycerol broth (25% v/v). For the day to day experiments, the bacteria were maintained on nutrient agar plates at 4°C in a refrigerator and sub-cultured at an interval of two weeks.

Screening for the efficient oil degrading bacteria

A crude oil biodegradation experiment was carried out to select the

efficient oil degraders for a detailed examination. The 44 isolated bacteria were each grown in a culture flask of 250 ml capacity containing 99 ml of mineral salts medium supplemented with sterile crude oil (1 ml) as the sole source of carbon and energy. For each of the selected pure isolates, a bacterial colony was pre-grown in 5 ml of peptone water for 24 h before seeding into the culture flasks. Control flasks were added and subjected to the same experimental conditions as the samples, except for the absence of bacterial culture. The experiments were carried out in two replicates for each isolate and the inoculated flasks were incubated in an orbital shaker at room temperature (25±2.0°C) at 150 rpm for five days. The concentration of crude oil in the culture fluids was analyzed using a GC/MS machine before inoculating the pure isolates and after five days of incubation. The isolates which showed better crude oil degradation during the five days of incubation were selected for a detailed analysis.

The selected bacterial isolates (Ngara-T1, Kig3-T4, Kig5-T2, Kas2-T7, Kig5-1, Kig3-T3, Kas2-T5 and Gat-3) were incubated for 21 days in culture bottles of 500 ml capacity containing 198 ml of enrichment medium supplemented with 2 ml of sterile crude oil. The residual oil in the culture fluids was extracted with chloroform every week and analyzed using a GC/MS. The residual oil was extracted by pipetting 10 ml aliquots of thoroughly shaken culture fluids into a separating funnel of 100 ml capacity followed by10 ml of chloroform. The funnel was vigorously shaken for one to two minutes and then allowed to settle for two minutes for the aqueous and organic phases to separate. The oil-solvent layer was removed using a separator funnel and the solvent was evaporated using a water bath at 40°C. The oil was reconstituted to 0.5 ml with chloroform and 1 µL of the extracted crude oil was analyzed by GC/MS.

Analysis of un-degraded crude oil by GC/MS

The total petroleum hydrocarbons were analyzed according to the method by Wongsa et al. (2004) with a GC/MS (GC model 6890N and MS model 5975 - Agilent Technologies, USA). The GC/MS was equipped with a column from Agilent Technologies (19091 S-433) comprising of a length of 30 M, internal diameter of 0.25 mM and film thickness of 0.25 µM. The injector temperature was set at 250°C, while the column temperature was first set at 40°C and held for three minutes before raising it to 220°C at a rate of 5°C per minute. The total area under the resulting chromatograms were noted and the chromatograms were analyzed by an automatic mass spectral deconvolution and identification system (AMDIS) to identify the petroleum components and compared against the National Institute of Standards and Technology (NIST) library in the GC/MS.

Analysis of Biodegradation data

The percentage of total hydrocarbons un-degraded after a given time was calculated by comparing the area of the peaks with that of the corresponding peaks shown by a control that was subjected to the same experimental conditions as the samples, except for the absence of a bacterial culture (Wongsa et al., 2004). Statistical analysis of variance was done at a 95% confidence limit to compare degradation efficiency among the isolates during the 21 days of incubation.

Bacterial genomic DNA extraction

The genomic DNA of the efficient oil degrading bacteria was

extracted by the CTAB method as described by William and Copeland (2004). The isolates were each grown in 10 ml of peptone water for overnight and 3 ml of the bacterial culture were transferred to a 15 ml tube and centrifuged at 10,000 rpm for five minutes. The supernatant was discarded and the cells were resuspended in 1 ml of Tris-EDTA (TE) buffer and transferred to a clean centrifuge tube of 1.5 ml. 40 µL of lysozyme were added, mixed and solution incubated for 30 min at 37°C. 40 µl of 10% sodium dodecyl sulphate (SDS) were added and mixed; and then 8 µL of 10mg/ml Proteinase K were added, mixed and mixture incubated for 1hr at 56°C. 100 µL of 5M sodium chloride were added and mixed. 100µL CTAB/NaCl were added, mixed and incubated at 65°C for 10 min. 0.5 ml of Phenol: chloroform: isoamyl (25:24:1) were added, mixed and spun at maximum speed for ten minutes at room temperature. 0.5ml of chloroform: isoamylalcohol (24:1) were added, mixed well and spun at 13,200 rpm at room temperature. 500 µL of the aqueous phase were transferred to a clean centrifuge tube and 3 volumes of cold absolute ethanol at ₋20°C were added followed by 10% of 3 M sodium acetate and precipitated overnight at ₋20°C. The tubes were spun at 13,200 rpm for 30 min, at 4°C and the supernatant was discarded. The pellet was washed with 500 µL of cold ethanol (70%), spun at 13,200 rpm for 15 min; and the supernatant was discarded. The pellet was dried at room temperature for 20 min and then eluted with 50 µL of the TE buffer. 0.2 µL of RNAse were added, mixed and incubated at room temperature for one hour, and the extracted DNA was stored at ₋20°C till used.

Genomic amplification of 16S rRNA

The 16S ribosomal RNA gene for the selected oil degrading bacteria was amplified by PCR using universal primer pair; 16S rRNA forward 5'-GAGTTTGATCCTGGCTCAG-3' and 16S rRNA reverse 5'-AAGGAGGTGATCCAGCC-3' (Wongsa et al., 2004), which correspond to positions 9 to 27 and 1525 to 1541, respectively, in the 16S rRNA gene sequence of *Escherichia coli* (Wongsa et al., 2004). The DNA was amplified using a Taq PCR kit (Thermo Scientific,® Finland) containing the following stock solutions; 10× PCR buffer, 25 mM of MgCl₂, 10 mM each of dNTPs, 5 U/µL of *Taq* polymerase and 10 µM of each primer. The amplification was done in a final volume of 50 µL containing 5 µL of genomic DNA. The conditions for amplification consisted of 30 cycles of 94°C (30 s), 52°C (30 s) and 72°C (30 s), plus one additional cycle with a final five minute chain elongation. All amplifications were performed in a GeneAmp PCR System (model 9700, Applied Biosystems,® USA). The PCR products were separated on agarose gel electrophoresis (1%) and the sizes of the fragments were estimated based on a 50 base pair DNA ladder (Sigma,® USA).

DNA Sequencing

The PCR products were purified and submitted to Inquaba Biotechnical Industries in Pretoria, South Africa, for sequencing.

Analysis of sequence data

The isolates were identified by comparing the 16S rRNA consensus sequence data with those in the National Center for Biotechnology Information (NCBI) GenBank data bases using the n-BLAST program Sequences with more than 98% identity with a GenBank sequence were considered to be of the same species (Kumar et al., 2006).

Amplification of *xylE* gene for biodegradation efficiency

The *xylE* gene was amplified by PCR in the selected oil degrading bacteria using the primer pair; DEG-Forward 5'-CGACCTGATCATCGCATGACCGA-3' and DEG-Reverse 5'-TCTAGGTCAGTACACGGT CA-3' which plays a key role in the metabolism of aromatic rings of aromatic compounds (Malkawi et al., 2009). Genomic DNA was amplified using a Taq PCR kit (Thermo Scientific®, Finland) containing the following stock solutions; 10× PCR buffer, 25 mM of MgCl₂, 10 mM of each dNTPs, 5 U/µL of *Taq* polymerase and 10 µM of each primer. The amplification was done in a final volume of 25 µL containing 2.5 µL of genomic DNA. The PCR conditions were; 30 cycles of 94°C (30 s), 50°C (30 s) and 72°C (30 s), plus one additional cycle with a final 5 min chain elongation. All amplifications were performed in a GeneAmp PCR System (model 9700, Applied Biosystems®). The PCR products were separated on agarose gel electrophoresis (1%) and the sizes of the fragments were estimated based on a Sigma 50 base pair DNA ladder (Sigma, USA) and the presence of *xylE* gene in the selected oil degrading bacteria was related to their biodegradation efficiency.

RESULTS

Phenotypic identity of oil degrading bacterial isolates

Mixed colonies of bacteria grew on the surface of petroleum agar plates and some colonies formed depressions on the petroleum agar plates (Figure 1). A total of 44 crude oil degrading bacteria were isolated from the soil samples. Based on their colonial and cellular morphology and biochemical characteristics, the bacterial isolates were categorized into eight (8) groups. The colony characteristics of the isolates are summarized in Table 1. The cell morphology and biochemical characteristics; and the number of isolates in each of the eight groups are shown in Table 2. The genera *Corynebacterium* and *Pseudomonas* were the most common in the soils contaminated with crude oil, followed by *Moraxella*, *Bacillus* and *Enterobacteriaceae*. The genera *Nocardia, Serratia* and *Rhodococcus* were the least abundant isolates. Some isolates (23) could not be placed in a particular genus due to the phenotypic characteristics they exhibited and therefore they remained un-identified.

Oil biodegradation activity of the bacterial isolates

From the crude oil biodegradation experiment that was initially carried out (for five days), the bacterial isolates that degraded over 20% of the crude oil in the liquid medium (Figure 2) were selected for a detailed analysis. A relatively higher degradation activity was exhibited in the test bottles than in the negative control set-up.

The selected strains degraded the total petroleum hydrocarbons to over 60% by the end of the third week of incubation; and in all experiments inoculated with bacteria, the percentage of oil degradation was observed to increase with increase in the incubation period (Figure 3).

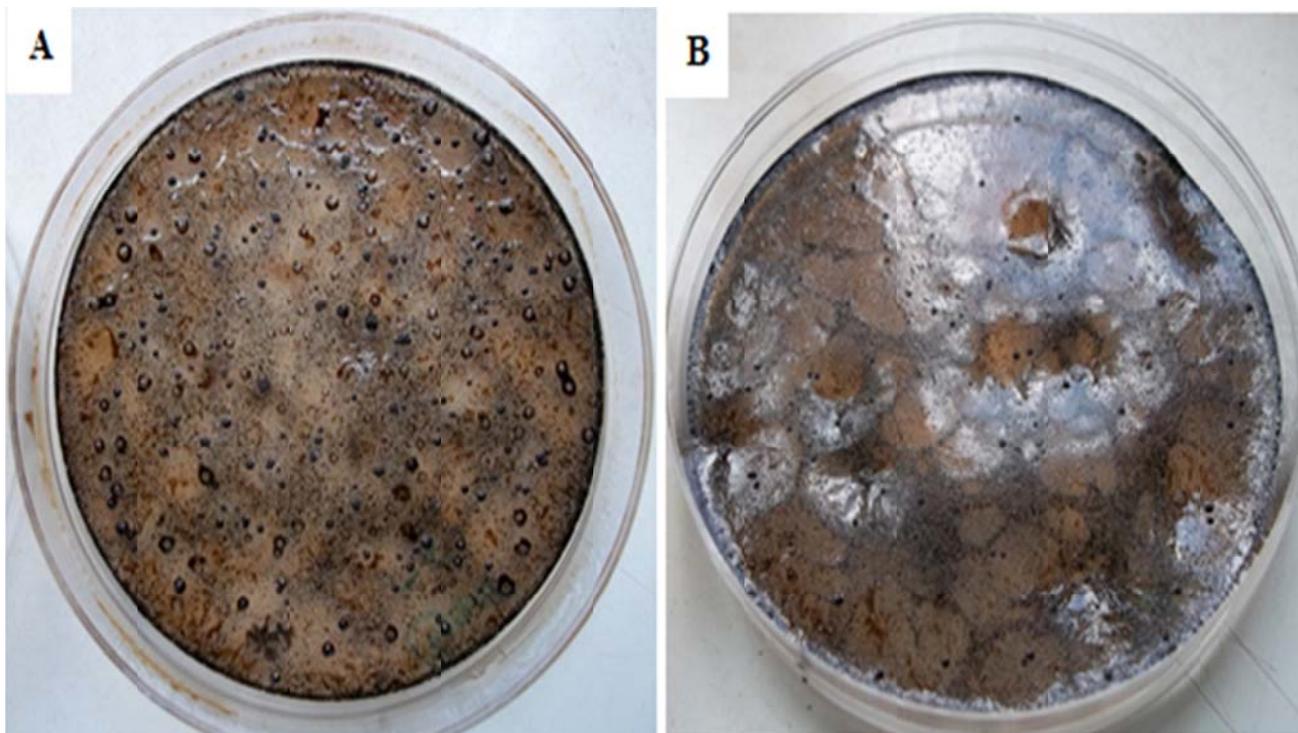Among the eight isolates analyzed, Kig3-T4 and Ngara-

Figure 1. Appearance of the oil degrading bacterial colonies on petroleum agar plates. Arrows show the regions of bacterial colonies. The dark appearance in the plates was due to the crude oil mixed with agar medium. Plate A – The arrows indicate bacterial colonies surrounded by a dark film of crude oil on surface of petroleum agar plate; Plate B – The arrows indicate bacterial colonies formed in depressions on the surface of petroleum agar plate.

T1 exhibited the best degradation activity (91.9 and 94.2%, respectively) followed by isolate Kig5-T2 and Kas2-T7 (89.2 and 83.2% respectively). These were followed by Kig5-1, Kig3-T3 and Kas2-T5 with a biodegradation of 79.9%, 73.1 and 77.0% respectively. Gat-3 was the least crude oil degrader with 67.2% as shown in Figure 3. The rate of hydrocarbon degradation in the control experiment was relatively low (29.7%) as compared to the rest of the experiments inoculated with bacterial isolates.

The statistical analysis of variance revealed that the percentage of oil degradation at the seventh day of incubation, was not statistically significant ($P>0.05$) for Gat3. It was, however, significant ($P<0.05$) for Kas2-T7 and Kig3-T3; very significant ($P<0.01$) for Kas2-T5, Kig5-T2 and Kig5-1; and extremely significant ($P<0.001$) for Kig3-T4 and Ngara-T1. During the 14th day of incubation, the degradation was very significant ($P<0.01$) for Gat3; and extremely significant ($P<0.001$) for Kas2-T7, Kas2-T5, Kig5-T2, Kig5-1, Kig3-T3, Kig3-T4 and Ngara-T1. The degradation at the 21st day of incubation was extremely significant ($P<0.001$) for Gat-3, Kas2-T7, Kas2-T5, Kig5-T2, Kig5-1, Kig3-T3, Kig3-T4 and Ngara-T1.

The GC/MS analysis of the crude oil sample revealed both aliphatic chain hydrocarbons (C9 to C24) and poly-cyclic aromatic hydrocarbons such as; o-*xyl*ene, cyclo-hexane, benzene, 1-ethyl-3-methyl, benzene 1, 2, 4-trime-thyl and benzene 1,3,5-trimethyl in the crude oil sample (Figure 4). As indicated in Figure 5A2 and E2, the hydro-carbon components in the crude oil were reduced in abundance after the three weeks of incubation in experi-ments that were inoculated with bacteria; compared to a minimal reduction in the control experiment (Figure 5G1).

Amplification of *xylE* by PCR

The catabolic gene *xylE* was detected in two of the eight selected bacterial isolates; that is in lane A and C which corresponded to the isolates Ngara-T1 and Kig5-T2 respectively (Figure 6). The degradation of crude oil by these two isolates (Ngara-T1 and Kig5-T2) after three weeks of incubation was very close, that is 91.4 and 89.2% for Ngara-T1 and Kig5-T2 respectively. The isolate Kig3-T4 which recovered most of the crude oil (94.2%) and other isolates with moderate and least crude oil degradation percentages did not possess the gene *xylE*.

Genotypic Identification

The 16S rRNA gene sequences were accessed from the public GenBank data bases using the n-BLAST program. Ngara-T1 which was Gram positive cocci and indole

Table 1. The colonial characters used to identify some of the bacterial isolates.

Isolate identity	Colony colour	Colony size	Colony form	Colony elevation	Colon margin	Genus identity
Ngara-T4, Kig3-4, Kas2-T2, Gat-T5, Kig3-T3	Blue-green, beige brown	Medium	Irregular, circular	Slightly raised	Undulated	*Pseudomonas*
Gat-3, Kig5-1, Gat-1, Kig5-T3, Kig5-T1, Kas2-T5 Kas2-T4	Cream, grey, white	medium	Circular	Raised, convex	Entire	*Corynebacterium*
Ngara-T2, Kig3-T2	Beige	Small	Punctiform	Convex	Entire	*Moraxella*
Ngara-3 Kig5-T2	Beige, cream	Medium	Irregular	Flat	Entire, undulated	*Bacillus*
Gat-T4	White, chalky	Small	Irregular	Raised	Entire	*Nocardia*
Ngara-T3, Kig5-T4	Cream	Medium	Circular	Convex	Entire	*Enterobacteriaceae*
Kig3-T1	Orange	Medium	Circular	Slightly elevated	Entire	*Serratia*
Kas2-1	White/buff	Medium	Circular	Flat	Entire	*Rhodococcus*

Table 2. The gram's staining and biochemical parameters used to identify the bacterial isolates.

| Isolate Identity	Number of Isolates	Gram's reaction and cell shape Test	Catalase Test	Oxidase Test	Indole Test	Glucose Ferm	Maltose Ferm	Lactose Ferm	Citrate Test	Urease Test	Haemolysis Test	Genus Identity
Ngara-T4, Kig3-4, Kas2-T2, Gat-T5, Kig3-T3	5	– rods	+	+	–	–	NA	NA	+	NA	NA	*Pseudomonas*
Gat-3, Kig5-1, Gat-1, Kig5-T3, Kig5-T1, Kas2-T4, Kas2-T5	7	+ rods	+	+ / –	NA	NA	–	NA	NA	NA	NA	*Corynebacterium*
Ngara-T2, Kig3-T2	2	– cocci	+	–	NA	NA	NA	NA	NA	–	+	*Moraxella*
Ngara-3 Kig5-T2	2	+ rods	+	NA	NA	+	NA	NA	NA	NA	NA	*Bacillus*
Gat-T4	1	+ rods	–	–	NA	NA	NA	NA	NA	NA	NA	*Nocardia*
Ngara-T3, Kig5-T4	2	– rods	+/ –	+ / –	NA	NA	NA	+	–	–	NA	*Enterobacteriaceae*
Kig3-T1	1	– rods	–	–	–	+	NA	–	NA	NA	NA	*Serratia*
Kas2-1	1	+ cocci	–	+	NA	+	NA	NA	NA	NA	NA	*Rhodococcus*
Ngara-1, Ngara-2, Gat-T3, Ngara-4, Ngara-T1, Gat-2 Gat-T1, Gat-T2, Kig5-2, Kig5-3, Kig5-4, Kas2-2, Gat-4, Kig3-3, Kas2-3, Kas2-4, Kas2-T1, Kas2-T3, Kas2-T6, Kas2-T7, Kig3-1, Kig3-2, Kig3-T4,	23	+ / –	+ / –	+ / –	NA	NA	NA	NA	NA	NA	NA	Un-identified

NA, Not applicable; Ferm, fermentation
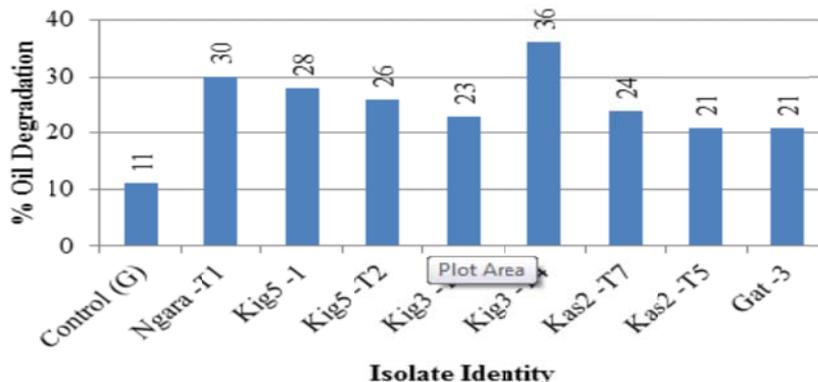
Figure 2. Crude oil degradation by the bacterial isolates during the five days of incubation in MSM supplemented with crude oil (1%) as the sole source of carbon. The experiments were incubated aerobically in duplicates on a rotary shaker (150 rpm) at room temperature (25 ± 2.0°C). Isolates that exhibited a relatively higher degradation (over 20%) were selected for a detailed analysis.
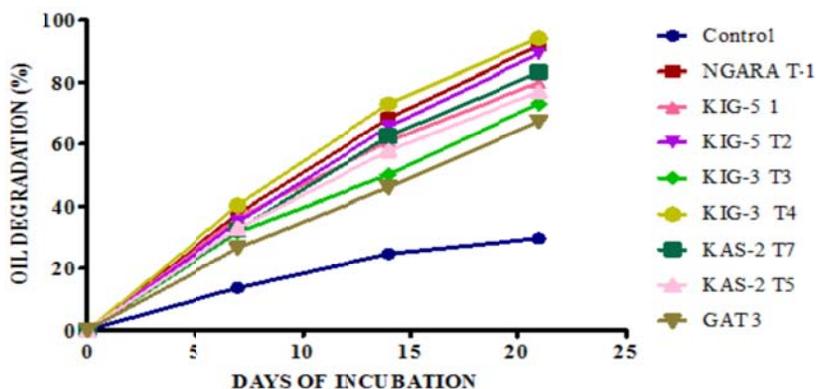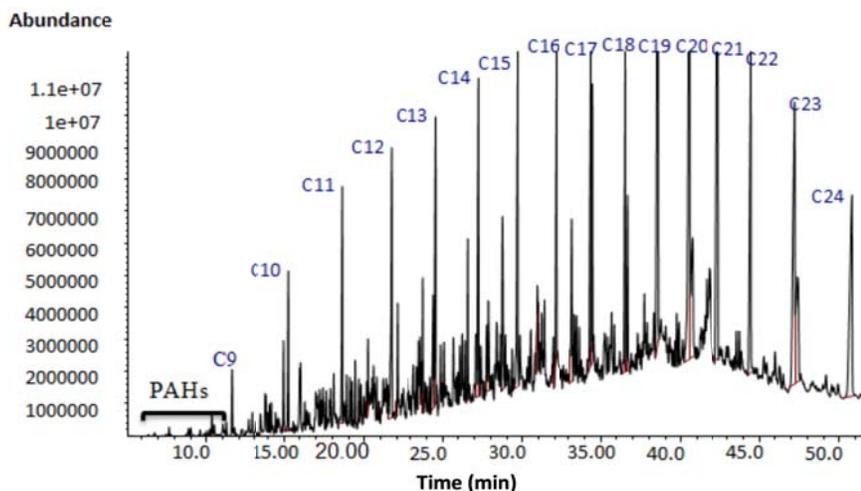

Figure 3. The percentage of degraded crude oil by selected bacterial isolates during three weeks of incubation. The experiments were incubated aerobically in duplicates on a rotary shaker (150 rpm) at room temperature (25 ± 2.0°C).

Figure 4. The crude oil degradation profiles as revealed by GC-MS analysis. The abundance of the polycyclic aromatic hydrocarbons (PAHs) was relatively low as compared to the aliphatic hydrocarbons (C9-C24).
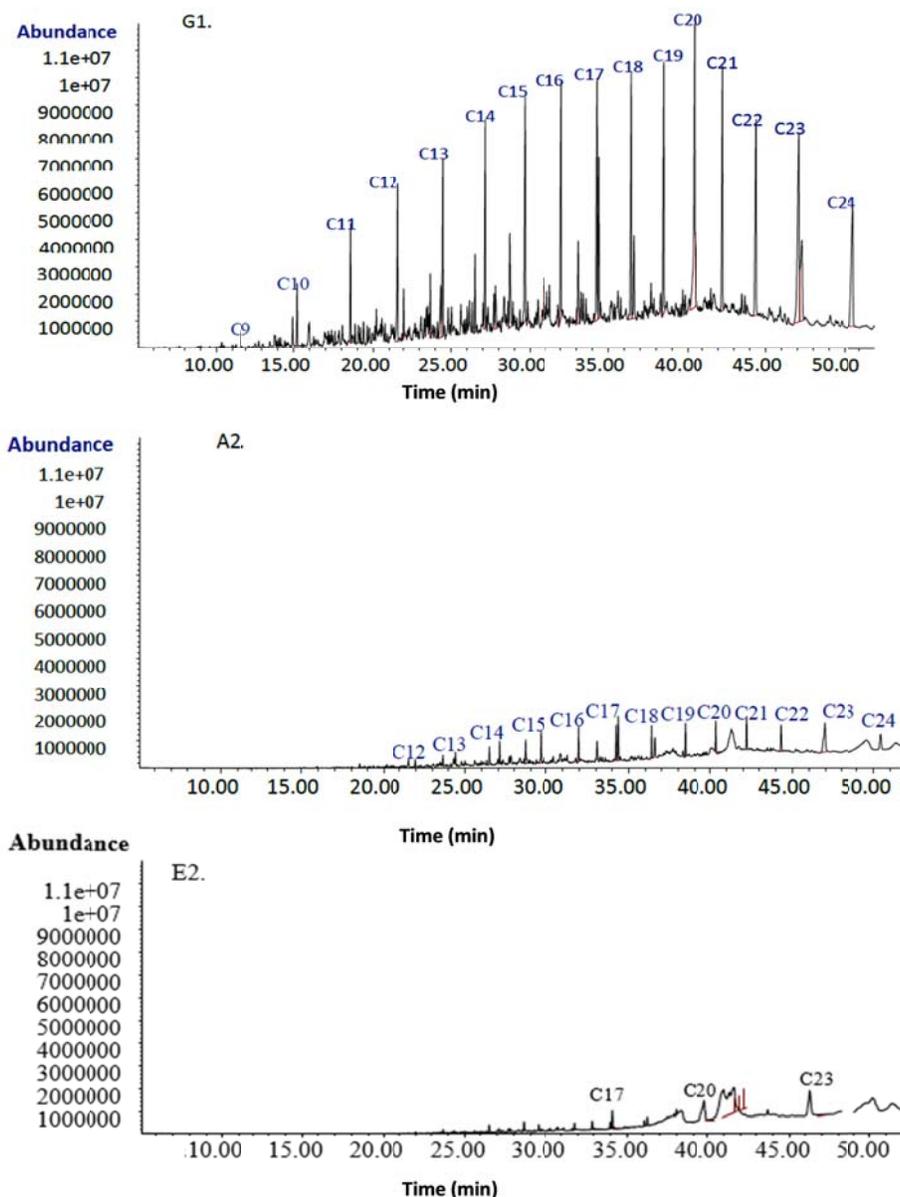
Figure 5. The GC/MS profile of crude oil components in culture medium after three weeks of incubation. G1, Control experiment (without inoculum). A2, hydrocarbon degradation by Ngara-T1. E2, hydrocarbon degradation by Kig3-T4. All PAHs and most aliphatic hydrocarbons were degraded in experiment A2 and E2 by the inoculated bacteria whereas they were still detectable in G1.

negative was 99% identical to *Stenotrophomonas maltophilia,* suggesting that Ngara-T1 was a strain of this species. Kig5-1 was Gram negative rod and positive for oxidase and catalase as it is for *Burkholderia* sp.; and it was 94% identical to *Burkholderia* sp., suggesting that Kig5-1 was a strain of *Burkholderia* sp. The strain Kig5-T2 was 82% identical to *Delftia tsuruhatensis* and the phenotypic characters were Gram negative rods, oxidase positive and catalase negative, similar to *Delftia tsuruhatensis*; suggesting that Kig5-T2 was *Delftia*

tsuruhatensis. The isolate Kas2-T5 was 95% identical to *Curtobacterium* sp. and its taxonomic characteristics were similar to *Curtobacterium* sp.; that is, Gram positive rods, positive for oxidase and catalase. These characters suggest that Kas2-T5 was *Curtobacterium* sp.

The alignment for Kas2-T7 was 98% identical to that of *Acinetobacter junii*. Kas2-T7 was Gram positive cocci, oxidase negative and catalase positive as it is for *Acinetobacter* sp., implying that Kas2-T7 was *Acinetobacter junii*. Similarly, the sequence alignment for
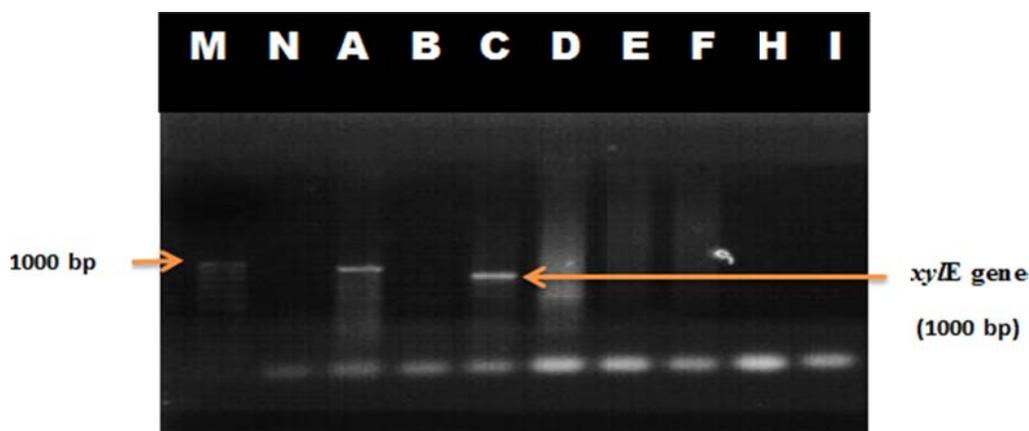
Figure 6. PCR amplification of the catabolic gene *xylE* in the selected isolates. M is the DNA marker (bands not clearly visible), N is the negative control (without DNA) and A, B, C, D, E, F, G and H lanes loaded with DNA from the selected isolates; Ngara-T1, Kig5-1, Kig5-T2, Kig3-T3, Kig3-T4, Kas2-T7, Kas2-T5 and Gat-3 respectively. The catabolic gene *xylE* was detected in lane A and C.

Table 3. The 16S rRNA gene sequence of the selected isolates in compulsion to the 16S rRNA gene sequences in the public gene Bank data base.

Isolate identity	Genus identity	Genotypic identity	Querry cover (%)	Max Identity (%)	Accession number
Ngara-T1	Un-identified	*Stenotrophomonas maltophilia*	100	99	EU741084.1
Kig5-1	*Corynebacterium*	*Burkholderia* sp.	98	94	JX845723.1
Kig5-T2	*Bacillus*	*Delftia tsuruhatensis*	95	82	AY684785.1
Kig3-T3	*Pseudomonas*	*Pseudomonas aeruginosa*	100	99	NR_074828.1
Kig3-T4	Un-identified	*Uncultured Acinetobacter* sp.	100	87	FJ191646.1
Kas2-T7	Un-identified	*Acinetobacter junii*	100	98	HE651918.1
Kas2-T5	*Corynebacterium*	*Curtobacterium* sp.	100	95	KC466122.1
Gat-3	*Corynebacterium*	*Paenibacillus agaridevorans*	99	95	NR_025490.1

Kig3-T4 was 87% identical to *Acinetobacter* sp. and the strains were Gram positive cocci and oxidase negative as it is for *Acinetobacter* sp. Kig3-T4 had some resemblance to *Acinetobacter* sp.

Gat-3 was a strain of *Paenibacillus agaridevorans*. The sequence alignment for Kig3-T3 was 99% identical to that of *Pseudomonas aeruginosa* and its taxonomic charac-teristics were typically the same. The isolates of Kig3-T3 were Gram negative rods, oxidase positive/negative, catalase positive and with production of a greenish pigmentation, which suggests that Kig3-T3 was a strain of *Pseudomonas aeruginosa.* The BLAST search for the 16S rRNA consensus sequence of the selected isolates are summarised in Table 3 (Figure 7).

In this study, strains of *Pseudomonas* sp. were among the most numerous crude oil degrading bacterial isolates, and were selected among the isolates that demonstrated relatively higher crude oil degradation.

DISCUSSION

In the present research, crude oil degrading bacteria were isolated from the oil exploration areas in Western Uganda. Such organisms are naturally present in these environments and play a very important role in the removal of the pollutants (Obayori et al., 2012). The crude oil degrading bacteria were phenotypically ident-ified as belonging to the genera; *Corynebacterium, Pseudomonas, Moraxella, Bacillus, Enterobacteriaceae, Nocardia, Serratia* and *Rhodococcus.* These genera have previously been reported as containing oil degrading species (Bicca et al., 1999; Iwabuchi et al., 2002; Malkawi et al., 2009; Obayori et al., 2012; Rajaei et al., 2013; Wongsa et al., 2004).

In this study, GC/MS analysis revealed hydrocarbon components with carbon numbers as high as C24. All components of the crude oil were biodegraded and
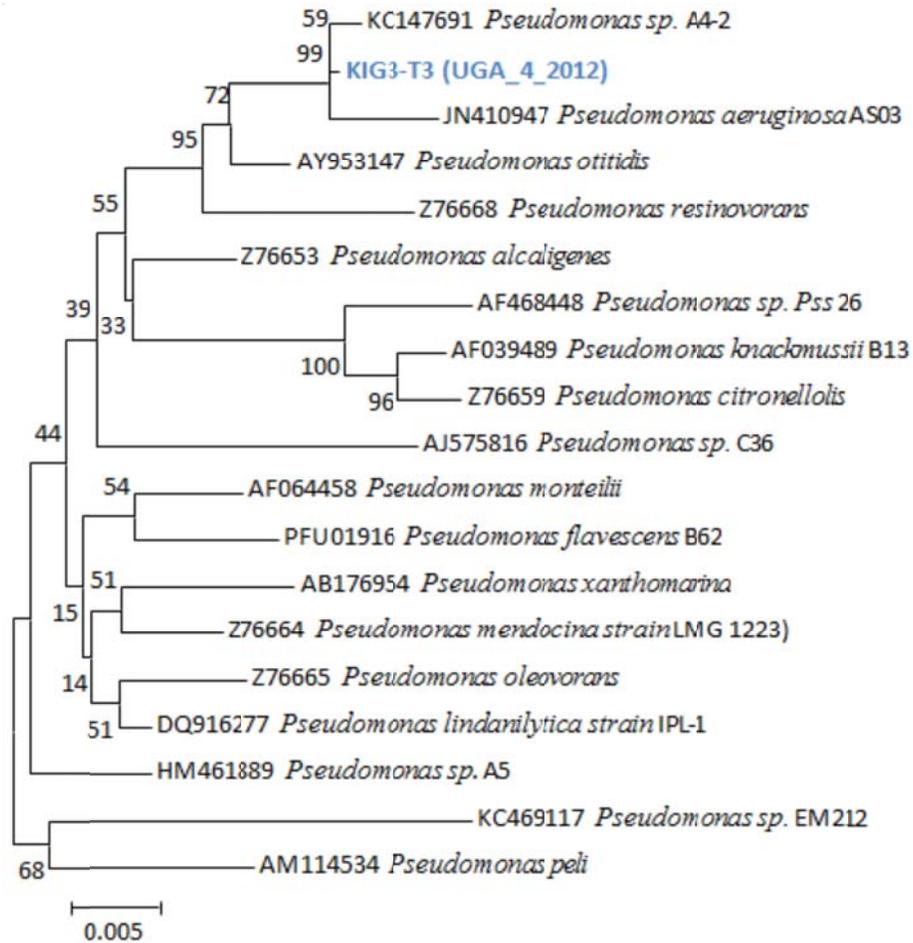
Figure 7. The phylogenetic tree showing isolate Kig3-T3 and its related Pseudomonas sp. The tree was constructed on the basis of 16S ribosomal RNA gene sequence using the neighbor-joining method. The evolutionary analyses were conducted using MEGA5 software. The analysis indicated that the closest relatives of strain Kig3-T3 were Pseudomonas sp. Strain A4-2 and Pseudomonas aeruginosa strain AS03.

reduced by the bacterial isolates as compared to the control. The crude oil was the sole source of carbon and energy in the liquid growth medium (MSM), indicating that these strains were capable of degrading and utilizing crude oil hydrocarbons. This is in agreement with a previous study by Wongsa et al. (2004) that observed a reduction in the hydrocarbon quantities after incubation of the oil bio-degrading bacteria in a diesel enriched mineral salts medium. The crude oil was dissolved into the culture broth in the experiments inoculated with bacteria, while it remained suspended above the Un-inoculated broth in the control experiment, suggesting that the bacteria may have produced biosurfactants and enzymes which emulsified the crude oil. Production of bio-surfactants is one of the ways the microbes can take up hydrophobic substrates and it has been reported in organisms such as *Pseudomonas aeruginosa* Acinetobacter sp. and Bacillus polymyxa (Rajaei et al., 2013).

The degradation of hydrocarbons is aided by catabolic genes carried by the oil degrading organisms, and identification of such genes is commonly performed to evaluate the microbes for bioremediation processes (Rajaei et al., 2013). In this study, the catabolic gene *xylE* was detected in two of the eight selected bacterial isolates; that is, Ngara-T1 and Kig5-T2. The total petroleum hydrocarbons degraded by these two isolates (Ngara-T1 and Kig5-T2) was very close; that is, 91.4 and 89.2%, respectively, suggesting that presence of *xylE* gene may have played a similar role in the degradation of crude oil hydrocarbons in the two bacterial isolates.

However, isolate Kig3-T4, which degraded most of the crude oil in the liquid medium (94.2%); and the other isolates with moderate and least crude oil degradation did not possess the catabolic gene *xylE*. This suggests that the presence of *xylE* gene is not the sole factor that contributes to crude oil degradation by bacteria. There

are seven catabolic genes that encode enzymes involved in a variety of known bacterial hydrocarbon degradative pathways that have been reported (Malkawi et al., 2009).

According to Harayama and Rekik (1989), hydrocarbon degrading bacteria possess oxygenases which catalyze reactions involving both biosynthesis and biodegradation. Such enzymes are classified into two groups: dioxygenases that catalyze the incorporation of both atoms of oxygen into substrates; and monooxygenases that catalyze the insertion of one atom of oxygen. The enzyme catechol 1,2-dioxygenase, cleaves the aromatic ring of catechol and the substrates of the ring-cleavage dioxygenases usually contain two hydroxyl groups on two adjacent aromatic carbons. The degradation of alkanes involves formation of an alcohol, an aldehyde and a fatty acid, which is cleaved, releasing carbon dioxide and forming a new fatty acid that is two carbon units shorter than the parent molecule in a process known as beta-oxidation (Malatova, 2005). *Acinetobacter* sp. possesses an alkane monooxygenase which oxidizes the second carbon atom leading to the production of a secondary alcohol; and the subsequent ketone is further metabolized to a primary alcohol for further breakdown (Malatova, 2005).

The alignment of the 16S rRNA gene sequence of the selected isolates revealed that the strains were identical to *Stenotrophomonas maltophilia*, *Burkholderia* sp., *Delftia tsuruhatensis*, *Pseudomonas aeruginosa*, *Acinetobacter* sp., *Acinetobacter junii*, *Curtobacterium* sp. and *Paenibacillus agaridevorans*. Except for *Delftia tsuruhatensis,* the rest of these strains have commonly been isolated and reported as oil degrading species (Laurie and Lloyd-Jones, 1999; Rajaei et al., 2013; Urszula et al., 2009). *Delftia tsuruhatensis* first described in 2003, was isolated from activated sludge collected from a domestic wastewater treatment plant in Japan, and it was able to degrade various (hazardous) aromatic hydrogen carbon compounds (Preiswerk et al., 2011). The Gram negative, oxidase positive and catalase negative strains of *Delftia* sp. isolated in this study, were able to degrade crude oil in the liquid medium as the sole source of carbon and energy.

Pseudomonas sp. have frequently been reported as crude oil degrading bacteria; and so far, they are the most studied of all the hydrocarbon degrading bacteria (Mittal and Singh, 2009). It is not surprising that strains of *Pseudomonas* sp. were among the most numerous crude oil degrading bacteria identified in this research. The isolates were Gram negative rods, oxidase positive-/negative, catalase positive and with production of a greenish pigmentation.

A study in 2009 by Obuekwe and others identified *Paenibacillus* sp. as a prominent crude oil degrader in the Kuwait desert environment; and it was able to survive the prevalent high soil temperatures (40-50°C) (Ganesh and Lin, 2009). The same study also showed that both *Paenibacillus* sp. and *Stenotrophomonas* sp. were effect-

tive in degrading diesel up to 83%, making them prominent hydrocarbon degraders. In this study, a Gram positive cocci and indole negative *Stenotrophomonas maltophilia* was the most efficient crude oil degrader; while the Gram positive and catalase positive strains of *Paenibacillus* sp. identified in this study were not prominent crude oil degraders. The difference in crude oil degradation could be attributed to the differences in environments where the two strains were obtained.

Curtobacterium sp. were listed among the genera of hydrocarbon degrading bacteria which grow in the presence of PAHs or alkanes (Verania, 2008). Previous researchers reported isolating *Curtobacterium* sp., with crude oil degrading activity from rhizosphere-bound soil samples (Rajaei et al., 2013). Strains of *Acinetobacter* sp. have been shown to degrade the hydrocarbons in crude oil in many studies. These same studies demonstrated the ability of *Acinetobacter* sp. to utilize crude oil hydrocarbons in liquid media and to compete with indigenous soil microorganism (Hanson et al., 1997; Rajaei et al., 2013). Similarly, strains of *Acinetobacter* sp. which were able to degrade the crude oil in liquid media were isolated during this study.

Conclusion

The oil degrading bacteria that were encountered in the soils around the oil exploration fields in Western Uganda were identified as; *Stenotrophomonas maltophilia*, *Burkholderia* sp., *Delftia tsuruhatensis*, *Pseudomonas aeruginosa*, *Acinetobacter* sp., *Acinetobacter junii*, *Curtobacterium* sp. and *Paenibacillus agaridevorans*. The oil degrading bacteria showed diverse and varying capacities to degrade the crude oil; with some degrading up to 90% and can be exploited for cleaning up hydrocarbon contaminated sites. There is need for more research on these isolates to establish whether it is possible to carry out crude oil bioremediation either through inoculation of specifically isolated microbial communities with higher degradation potential or targeted stimulation of the indigenous soil microbes. The catabolic gene *xylE* was not detected in all of the isolates that exhibited oil degrading because it is not the only gene that plays a role in hydrocarbon degradation by microbes. Hence, presence of other genes associated with this activity need to be investigated. To the best of our knowledge, this study on isolation, identification and determination of crude oil degradation efficiency of the bacteria was the first of its kind in Uganda. Therefore, the study is an important step towards the development of bioremediation strategies for cleaning of sites contaminated with oil pollutants and wastes. There is need to do further research in order to test the degradation activity of the isolates in combination and establish the safety of the isolates and of the by-products of oil metabolism in the environment.

Furthermore, there is need to screen for biosurfactant production, investigate mechanism of oil reduction and screen for other reported genes to establish the mechanism of biodegradation. There is also need to determine the optimum growth requirements of the isolates, test oil biodegradation in the field by the microbes and compare the isolates with well-known characterised organisms.

Conflict of Interests

The author(s) have not declared any conflict of interest.

Authors' contributions

Andrew Wedulo contributed to the conception of the idea, design, data collection, laboratory analysis and writing of the manuscript. David Kalenzi Atuhaire contributed to laboratory analysis, data analysis and writing of the manuscript. Sylvester Ochwo contributed to data collection, laboratory analysis and writing of the manuscript. Vincent Muwanika contributed to the conception of the idea, design and writing of the manuscript. Abel John Julian Rwendeire contributed to the conception of the idea and writing of the manuscript. Jesca Lukanga Nakavuma contributed to the conception of the idea, design, data analysis and writing of the manuscript. All read and approved the manuscript.

Conflict of Interest

The author(s) have not declared any conflict of interests.

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