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Research article

In vitro assessment of crude oil degradation by *Acinetobacter junii* and *Alcanivorax xenomutans* isolated from the coast of Ghana

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ABSTRACT

This study was aimed at using *in vitro* microcosm experiments to assess crude oil degradation efficiency of *Acinetobacter junii* and *Alcanivorax xenomutans* isolated along Ghana's coast. Uncontaminated seawater from selected locations along the coast was used to isolate bacterial species by employing enrichment culture procedures with crude oil as the only carbon source. The isolates were identified by means of the extended direct colony transfer method of the Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectroscopy (MALDI-TOF MS), as *Acinetobacter junii*, and *Alcanivorax xenomutans*. Remediation tests showed that *Acinetobacter junii* yielded degradation efficiencies of 27.59 %, 41.38 % and 57.47 %. Whereas efficiencies of 21.14 %, 32.18 % and 43.68 % were recorded by *Alcanivorax xenomutans* representing 15, 30 and 45 days respectively. Consortia of *Acinetobacter junii*, and *Alcanivorax xenomutans* also yielded 32.18 %, 48.28 % and 62.07 % for the selected days respectively. Phylogenetic characterization using ClustalW and BLAST of sequences generated from the Oxford Nanopore Sequencing technique, showed that the Ghanaian isolates clustered with *Alcanivorax xenomutans* and *Acinetobacter junii* species respectively. An analysis of the sequenced data for the 1394-bp portion of the 16S rRNA gene of the isolates revealed >99 % sequence identity with the isolates present on the GenBank database. The isolates of closest identity were *Alcanivorax xenomutans* and *Acinetobacter junii* with accession numbers, NR_133958.1 and KJ147060.1 respectively. *Acinetobacter junii* and *Alcanivorax xenomutans* isolated from Ghana's coast under pristine seawater conditions have therefore demonstrated their capacity to be used for the remediation of crude oil spills.

1. Introduction

Globally, the exploitation of petroleum hydrocarbons from the offshore oil and gas sector have contributed to the incidence of marine oil spills. The natural marine environments are seriously damaged in crude oil-contaminated areas, lasting decades [1]. Long-lasting and environmentally friendly methods of research on oil removal are pivotal for mitigating oil spill hazards and ensuring environmental sustainability. In recent times, physical or chemical approaches are employed at early stages of oil spill as the first

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choice in emergency response with its associated high cost [2]. Traditional methods of remediation such as oil fences and skimmers are capable of eliminating oil spills to an extent, but not completely [3]. Other approaches such as dispersants and gelling agents usually lead to secondary pollution and in some cases increased toxicity [4]. An increasing number of scientists have therefore turned their attention to bioremediation methods for petroleum hydrocarbon pollutant degradation [5]. Modern methods such as systems biology techniques, through bacteria engineering, have also been used to achieve successful biodegradation of many types of petroleum hydrocarbons. The technique has shown its immense potential for petroleum hydrocarbon degradation in metabolic engineering through the modification of cellular metabolism to achieve desirable biochemical pathways. Also, enzyme engineering which involves the alteration and optimization of enzymes to enhance their properties has also been used [6].

Primarily, hydrocarbons from petroleum are biodegraded with bacteria, fungi, and yeast with or without other amendments. Evidence from research has demonstrated that when applied alone, these organisms can degrade the petroleum hydrocarbons in the order; 0.13 %–50 % for soil bacteria, 0.003 %–100 % for marine bacteria and 6 %–82 % for soil fungi [7]. Other researchers have stated that varied microbial clusters possess wide enzymatic abilities capable of degrading complex, including recalcitrant parts of crude oil found in soil [8], fresh water and marine ecosystems [9].

In this research *Acinetobacter junii* and *Alcanivorax xenomutans* were used to degrade crude oil sourced from the Jubilee Oil Field in Ghana in a laboratory experimental spill. *Acinetobacter junii* and *Alcanivorax xenomutans* have been successfully used to degrade crude oil spills in other parts of the world [10,11].

It must be noted that successful biodegradation efforts of petroleum hydrocarbon pollutants began at the Gulf of Alaska and in Prince-William-Sound. These can be traced back to the 1980's, during the cleanup of oil spillage triggered by the oil tanker 'Exxon Valdez' in 1989. The success of the cleanup generated a great deal of interest in the possibility of applying technology involving biodegradation and bioremediation [7].

Other research and reviews conducted by several authors have shown that over the last 3 decades, petroleum hydrocarbon pollution in aquatic and terrestrial ecosystems has been considerably resolved employing bioremediation [12–15]. The authors augue that different types of microbes also exist for degrading various forms of hydrocarbons including aliphatics, monoaromatics, polyaromatics and resins.

A number of factors influence the degrading process, and these are important in choosing a suitable approach for any hydrocarbon pollutant. These factors comprise but are not restricted to type of bacteria, composition of the hydrocarbon, temperature, pH and nutrients (nitrogen and phosphorus) [7]. Therefore, degradation using microbes is the main natural mechanism for the environmental cleanup of petroleum hydrocarbon pollutants and is highly recommended [16].

The usage of microbes present at the site of pollution for the remediation of crude oil spills has become essential due to the numerous advantages it offers, including being friendly to the environment and cost effective. However, the recalcitrance of crude oil in the environment, attributable to their hydrophobic properties and their consequent high affinity to soil particles, limits their bioavailability. This is obviously a source of concern and will require urgent attention [17]. Again, limited exchange of substances at the interface of crude oil and water reduces the activity of microbes found in water that may be responsible for the breakdown of the crude oil. These limitations of the use of microorganisms have prompted other modifications to the approach. Emulsifiers have thus been used to break the boundary between the two phases to help increase solubility and availability of the crude oil for microbial action. Other processes such as biostimulation, bioaumentation, bacterial cell immobilization and application of bacterial consortia have been used to improve on the bioremediation methods [18,19]. In this study, *in vitro* microcosm experiments were conducted using bacteria isolated from the coast of Ghana. This study is therefore expected to provide further insights into the assessment of crude oil degradation using *Acinetobacter junii* and *Alcanivorax xenomutans* isolated from Ghana's coastline.

2. Methodology

2.1. Study design

The experiment was cross-sectional in design to test the degradative capacities of selected bacteria. *In vitro* bioaugmentation of microcosms was used as the study approach, in which the seawater was cleaned up from simulated crude oil spills, by means of native cultured bacteria within predetermined time limits.

2.2. Obtaining the crude oil samples

Samples of Jubilee crude oil were obtained from crude separation tanks on Floating Production Storage and Offloading platform (FPSO Kwame Nkrumah) in 2 L containers without any prior treatments and transported to the laboratory. This oil comprises crude oil from various sources in a given geographical setting, drilled at specific periods. The first commercial quantity of crude oil was pumped in December 2010. However, the batch utilized for this research was the 31st composite acquired in February 2016 [20]. A 1 L polyethylene bottle (KANE-EM Industries, Ghana) was used to collect the crude oil sample, labeled clearly without any prior treatments, and kept in the laboratory at room temperature.

2.3. Obtaining the bacteria strains

Two (2) bacteria strains (*Acinetobacter junii* and *Alcanivorax xenomutans*) were isolated at the Biotechnology Center (BTC) of GAEC. The bacteria strains obtained from samples of sea water were taken from Tema (5° 36′ N, 0° 1′ W) and Takoradi (4° 52′ N, 1° 45′ W). The bacteria strains belong to a collection of hydrocarbon-degrading bacteria from previous research [21] from Ghana's coastline. The

isolation of the bacteria was done using mineral salts medium (MSM) without carbon content as source of nutrients [22]. Enrichment culture techniques with natural sea water and crude oil (serving as the sole carbon source) were also employed.

2.4. Preparation of mineral salt medium (MSM) for bacterial culture

Modified standard operation protocol for the preparation of mineral salts medium (MSM) was adopted to provide nutrition for the isolation of the microbes [22]. The MSM and agar (with no carbon content) were formulated using some specific micro and macro nutrients (10 g NaCl; 0.42 g $MgSO_4.7H_2O$; 0.29 g KCI; 0.83 g KH_2PO_4; 1.25 g K_2HPO_4; 0.42 g $NaNO_3$) and mixed in 1 L distilled water. About 15 g of bacteriological agar was dissolved in 1 L of the MSM to create the mineral salt agar. A portable autoclave (Prestige Medical Portable Autoclave, Fisher Scientific, UK) was used to sterilize the media at a pressure of 15 pounds per square inch (psi) for 30 min before inoculation.

2.5. Isolation and selection of crude-oil-degrading bacteria

Bacteria were isolated using slightly modified enrichment culture techniques by Nocera et al. [23]. Inoculations were done using eight 250 ml Erlenmeyer flasks holding about 100 ml sterile MSM with 1 ml sea water and 1 ml crude oil. The mixture was agitated using an IKA vortex GENIUS 3 (USA) for a few seconds and an Avantor INCU-Line incubator, (USA) was utilized to store the blend at 28 ^{O}C for 7 days. After the 7 days of incubation, a new 100 ml MSM with 1 ml crude oil was used to culture a 1 ml portion of the previous culture and incubated using identical settings. Following 4 succeeding isolation cycles, bacteria cultures were streaked onto mineral salts agar laced with crude oil, using a flame sterilized inoculation loop and incubated again at 28 ^{O}C for 7 days. Clean colonies were picked afterwards and kept in nutrient broth for additional laboratory analysis.

2.6. Identification and confirmation of bacterial isolates by Matrix Assisted Laser Desorption Ionization Time of flight mass spectrometry (MALDI –TOF/MS) using the extended direct colony transfer method

A slightly modified version of the extended direct colony transfer method by Schulthess et al. [24] was adopted for the MALDI-TOF identification. Bacteria stored in nutrient broth (as described in previous section) was plated on nutrient agar for 24 h to obtain fresh growth. The fresh colony samples were given specific codes and transferred onto a MALDI biotarget plate and 2 spots used for each sample. One microliter formic acid (70 %) was then applied to the bacterial stains and set aside to dry at room temperature. Once the sample spots were dried, a 1 μL α-cyano-4-hydroxycinnamic acid (HCCA) matrix solution in 2.5 % trifluoroacetic acid (Bruker Daltonics) and 50 % acetonitrile was placed on top of each sample.

The resultant matrix spot was set aside to air-dry for 10 min at room temperature, where a homogenous mix was observed. After drying, the biotarget plate was inserted into the MALDI-TOF Mass Spectrometer. Measurements were then made using a MALDI Biotyper (MBT) Compass from Bruker Daltonik GmbH, Bremen-Germany.

Bruker Biotyper software was used in identifying the spectra obtained from the isolated bacteria by MALDI-TOF analysis. Bacterial identification was done by comparing the current spectra to those listed in the data genbank. Corresponding profiles were represented on a logarithmic scale with scores ranging from 0 to 3. The values were explained according to the manufacturer's instructions. Values ranging from 2.300 to 3.000 were used to suggest a highly possible species-level identification, 2.000–2.299 suggested genus level and 1.70–1.999 score were designated as probable genus level identification. Bacterial test standards from Bruker Daltonics, Bremen, Germany (Catalogue #255343) were used for the calibration [25]. The spotted target plates were then inserted into the MALDI-TOF equipment which generated mass spectra in the positive linear mode. A 200-Hz frequency-tripled nitrogen laser, shooting at 337 nm targeted the sample spots at a mass range of 2–20 kDa (kDa). Charged masses of bacteria generated from the lasers in the MALDI were accelerated at 20 kV at the Time-Of-Flight (TOF) using a grid at 19.3 kV at the field-free drift region, in a short linear manner to the detector. A flexAnalysis software (version 3.4, Bruker Daltonics) was employed to optimize baseline corrections and noise filtration on the spectra generated. Spectra from the MALDI-TOF MS investigation was compared to a commercial Spectral Archive and Microbial Identification System (SARAMIS) database (version 4.10, bioMérieux, Saint-Vulbas, France) for the isolated bacteria. This was achieved by employing the standard limits of pattern-matching algorithms. The matching of spectra patterns of bacteria were shown as log scale 0–3 (Zhu et al., 2015) and this was recorded in a laboratory note book.

2.7. Total plate count (TPC)

The spread plate technique using serial dilutions was adapted from Briestenská et al. [26] to quantify the total bacteria of each bacterial isolate used for the remediation test. Plates were supplemented with 5.0 mg/ml nystatin to suppress the growth of fungi to ensure that only bacteria colonies were counted. Duplicate plates were incubated at 28 °C for 24 h before the colonies were counted. One ml of the pure bacterial cultures was poured aseptically into 9 ml sterile distilled water (dilution blanks) contained in a test tube. The test tubes were well labeled with the appropriate dilution factors (extent of dilution), flamed and covered with an aluminum foil in duplicates. One (1) ml of the previously inoculated sterile distilled water was subsequently transferred into 9 ml of sterile distilled water using sterile pipetted in another test tube bearing the well labeled dilution factor, flamed and also covered with aluminum foil in duplicates. The content in each test tube was mixed briefly with a vortex equipment. This process was repeated 7 times creating a 1 in 10-fold in each succeeding dilution. Nutrient agar was prepared by mixing 27 g in 1 L of distilled water and used to prepare the agar. Petri dishes were then filled to about one-third full of the prepared molten nutrient agar and allowed to solidify. About 1 ml of the

inoculum from the serial dilutions was then transferred onto the agar and spread on the entire agar using a bent glass rod. The setup was then incubated at 28 °C for 24 h. Plate count assumes that each life bacterium will produce a single colony, although some bacteria can clump together. Therefore, the results were represented as colony forming units (cfu) per ml of the sample. To obtain a statistically reliable estimate of the quantity of bacteria present in the cultures, the plates containing bacteria between 30 and 300 cfu/ml were selected. Those containing more than 300 were labeled too numerous to count (TNTC).

The number of colonies per ml of each culture were calculated as.

Concentration (cfu/ml) = number of colonies per plates * reciprocal of dilution factor selected Eg. cfu/ml = 140 *10^5 = 1.4 * 10^7Where 140 = number of colony forming units.

10^5 = reciprocal of the 10^{-5} dilution.

In this work, where the set up was in duplicates, the cfu were counted in the 2 plates and divided by 2 to increase accuracy.

2.8. Bacterial DNA extraction

The Zymo DNA extraction kit (Zymo Research, USA) was used to extract genomic DNA from bacterial cultures following the manufacturer's instructions. The extraction was undertaken at the Biotechnology Center of the Biotechnology and Nuclear Agricultural Research Institute (Ghana Atomic Energy Commission).

About 100 μl of pure bacterial isolates in a broth was supplemented with about 400 μl of genomic DNA lysis buffer in the ratio 4:1. The resulting mixture was thoroughly mixed by vortexing using IKA vortex GENIUS 3 (USA) and set aside for 5–10 min at room temperature. The mixture was poured into a Zymo-Spin IC column, positioned in a collection tube and centrifuged for 1 min at 10,000 rpm using a Hettich universal 320 centrifuge, USA. The flowthrough together with the collection tube was jettisoned and the Zymo-Spin IC column transferred to a new collection tube with the addition of about 200 μl of DNA pre-wash buffer to the spin column. The resulting mixture was centrifuged for 1 min at 10,000 rpm using a Hettich universal 320 centrifuge, USA. Again, the spin column was supplemented with about 500 μl of genomic-DNA wash buffer and centrifuged for 1 min at 10,000 rpm. Subsequently, about 10 μl of DNA elution buffer was added to the spin column after the column was transferred to a clean microcentrifuge tube. Final elution of the DNA was done at top speed (15,000 rpm) for 30 s after an incubation period of 2–5 min at room temperature. Storage of the eluted DNA was done at −20 ^{O}C for additional study. A 1 % agarose gel was used to assess the condition of the extracted DNA using a high-performance ultraviolet transilluminator (UVP, Cambridge, UK) for viewing.

2.9. Sequencing and phylogenetic analysis

An Oxford Nanopore Sequencer from the Noguchi Memorial Institute, Accra was used to sequence the extracted DNA obtained from the bacteria cultures employing the manufacturers guidelines. About 33 nucleotide sequences were used for the analysis. Removal of all ambiguous positions was done for each sequence pair using the pairwise deletion option. A total of 1969 positions were obtained in the final dataset. Sequence alignment, editing and comparison with other published works was done using CLUSTAL W [27] and BLAST [28]. The Molecular Evolutionary Genetics Analysis (MEGA) 11 software was used for phylogenetic analysis of the individual sequences [29]. Calculation of distances was done using the Kimura correction in a pairwise deletion manner [30]. A phylogenetic tree was constructed with the Neighbor-joining (NJ) methods with 1000 bootstrap replications in the MEGA 11 software [29].

2.10. Crude oil biodegradation

2.10.1. In Vitro Degradation by Single Bacteria

A microcosm experiment was set up for a simulated crude oil spill on natural sea water at 15 days intervals for 45 days under laboratory conditions. Nine (9) 250 ml Erlenmeyer flasks (autoclaved at 121 °C at 15 lbs pressure) containing 100 ml sea water were laced with 1 % v/v sterile (uncontaminated) crude oil from Jubilee oil field. About 1 % v/v inoculum of selected single hydrocarbon utilizing bacteria (HUB) of known total plate count (TPC) was added to the test flasks with their duplicates and controls to test for contamination. Three (3) different sets of setup and controls were mounted. At the terminal days all bacterial activities were halted with the addition of 1 % v/v, 1.0 N HCl to the relevant flasks [13].

2.10.2. In Vitro Degradation by bacterial consortium

Another microcosm experiment was set up for a simulated spilt crude oil on sea water (using bacteria consortium) at 15 days intervals for 45 days under laboratory conditions. Nine (9) 250 ml Erlenmeyer flasks (autoclaved at 121 ^{O}C at 15 lbs pressure) containing 100 ml sea water were laced with 1 % v/v sterile crude oil from Jubilee oil field. About 0.5 % v/v inoculum each of two selected HUB was added to the test flasks with their duplicates and controls to test for contamination. Three (3) different sets of setup and control were mounted. At the appropriate days, all bacterial activities were halted with the addition of 1 % v/v, 1.0 N HCl to the relevant flasks.

2.11. Determination of total petroleum hydrocarbons (TPH) using gravimetric analysis

On each terminal day, the total weight of residual crude oil of each set was determined using gravimetric analysis, at intervals of 15, 30 and 45 days to establish the rate of breakdown by the selected bacteria [31].

2.11.1. Extraction of residual crude oil

The test flasks and control were taken through slightly modified methods of extraction and analysis of residual crude oil by Zhang et al. [31], to determine the amount of crude oil utilized by the bacteria. Test flasks were mixed with equal volumes (10 ml) of cyclohexane in a separatory funnel and shaken vigorously for 2 min to break the oil-water barrier into a single mixture. The stop cock used to cover the separatory funnel was opened in the laminar flow to release gas build-up. About 2 ml of the cyclohexane was used to rinse the Erlenmeyer flask employed for the remediation experiment to ensure that all the residual crude oil was poured into the separatory funnel. A waiting period of 20 min was allowed for the mixture, which resulted in 3 layers. The top layer was a mixture of cyclohexane and residual crude oil, the middle layer was cell debris and the bottom layer was aqueous solutions of water and biosurfactants in soluble form. The 2 bottom layers were separated out into a beaker by opening the tap at the base of the separatory funnel. The top layer containing the cyclohexane mixed with the residual crude oil was taken out in a pre-weighed Erlenmeyer flask through a 12.5 cm Whatman filter paper folded in a funnel. About 10 g of anhydrous sodium sulphate was placed in the funnel to remove moisture. About 2 ml of cyclohexane was used to rinse the sodium sulphate before filtration. The sodium sulphate was activated by heating with the help of an oven to 103 ^{O} C before being used for the study. About 5 ml of the cyclohexane was also used to rinse the separatory funnel and the tip of the glass funnel 2 times to ensure no residue got stuck to the side of the glass walls. The collected aqueous layer was poured back into the separatory funnel and the process repeated 2 more times to ensure that any oil that may have leaked through was accounted for.

2.11.2. Weighing of residual crude oil

The flask containing the cyclohexane and crude oil residue was put into a water bath to evaporate all the organic solvent. The Erlenmeyer flask was then placed in an oven for 2 min and placed in a desiccator for the temperature to reduce while ensuring that there was no moisture contamination. The flask surface was wiped with a clean cloth before weighing. The crude oil utilized by the bacteria was then calculated by the difference between the initial and residual weights of the crude oil. The rates were achieved gravimetrically by subtracting the mass of the residual total petroleum hydrocarbon (TPH) remaining after the microcosm experiments from the mass of 1 ml of the original crude oil sample used for the experiment.

2.12 Statistical Analysis: The existence of significant differences between the amount of TPH degraded by the bacteria was done using the student T test at a 0.05 significance level. The evaluation was done to ascertain whether major variations exist between *Acinetobacter Junii* and *Alcanivorax xenomutans* when applied singly. It was also used to assess significant differences between the combined bacteria species compared to their single applications. All the assessments were performed by means of the R computer software [32].

3. Results

3.1. Identification of bacterial isolates by MALDI–TOF MS

Results from the MALDI-TOF MS (Matrix Assisted Laser Desorption Ionization Time-Of-Flight Mass Spectroscopy) revealed that sample one and its duplicate gave scores >2. This implied a genus/species-level identification and comparing the spectra to the commercial Spectral Archive and Microbial Identification System (SARAMIS) database showed that the best match for the sample was *Acinetobacter junii*, confirming a species level identification. Similarly, sample two and its duplicate also gave scores >2. This also suggested a genus level or species level identification and its comparison to the same data base (SARAMIS) also indicated that the best match was *Alcanivorax xenomutans* (Table 1), confirming a species level identification.

3.2. Total plate count (TPC)

The Enumeration of the heterotrophic bacteria using the spread plate enumeration method was undertaken after 24 h of incubation at 28 °C. To obtain a statistically reliable estimates of the quantity of bacteria present in the cultures, the plates containing bacteria between 30 and 300 cfu/ml were selected. Those containing more than 300 were labeled too numerous to count (TNTC) and plates containing less than 30 colonies were labeled too few to count (TFTC). Results of the total plate count showed that at average salinity and pH values of 32.43 mg L^{-1} and 7.99 respectively, *Acinetobacter junii* reported 1.48 × 10^5 CFU/ml whiles *Alcanivorax xenomutans* reported 9.8 × 10^4 CFU/ml (Table 2), which were statistically reliable estimates of the quantity of bacteria present in the cultures.

3.3. Determination of total petroleum hydrocarbon degradation

In Vitro Degradation by Single Bacteria: The degradation rates for selected bacteria isolates were tested singly and in a consortium in the laboratory at 15 days intervals for 45 days (Table 3). The residual TPH levels showed a gradual steady increase in degradation rates from days 15–45 for the two scenarios. In the case of using only *Acinetobacter junii* for the TPH remediation, days 15, 30 and 45 yielded TPH values of 0.24 (27.59 %), 0.36 (41.38 %) and 0.50 (57.47 %) degradation respectively. Whereas, *Alcanivorax xenomutans* yielded

Table 1
MALDI-TOF identification of Isolates with Score Values.

Number of isolates	Organism Best Match	Score value	Second Best match	Score Value
2	*Acinetobacter junii*	>2	*Acinetobacter junii*	>2
2	*Alcanivorax xenomutans*	>2	*Alcanivorax xenomutans*	>2

Table 2
Coliform forming units (CFU) in 1 ml of bacteria.

Type of Bacteria			CFU/ml
	Salinity (32.43 mg L^{-1})	pH (7.99)	
Acinetobacter junii			1.48×10^5
Alcanivorax xenomutans			9.8×10^4

Footnote: Data on CFU/ml, salinity and pH values are presented as means of two replicate measurements.

Table 3
In vitro degradation by *Acinetobacter junii* and *Alcanivorax xenomutans*.

		Acinetobacter junii		*Alcanivorax xenomutans*	
Number of Days	Mass of 1 ml crude oil = 0.87	Mean TPH Degraded	Percentage (%) Degradation	Mean TPH Degraded	Percentage (%) Degradation
15		0.24	(27.59)	0.16	(21.14)
30		0.36	(41.38)	0.28	(32.18)
45		0.50	(57.47)	0.38	(43.68)

Footnote: Data on Total Petroleum Hydrocarbon (TPH) degraded are presented as mean of two replicate measurements.

0.16 (21.14 %), 0.28 (32.18 %) and 0.38 (43.68 %) for 15, 30 and 45 days respectively.

In vitro degradation of crude oil by a consortium of bacteria **(***Acinetobacter junii and Alcanivorax xenomutans)* are shown in Fig. 1 below. The average TPH Degraded by *Alcanivorax xenomutans* and *Acinetobacter Junii* as well as a combination of both over a period of 45 days showed that the TPH degraded increased over time. On the 15th day of the experiment, the maximum recorded TPH degraded was 0.28 g, achieved by combining both bacteria into a consortium. Whereas the least degradation recorded was 0.16 g by *Alcanivorax xenomutans* while *Acinetobacter Junii* degraded 0.24 g of the TPH on average. On the 30th day of the experiment, the average TPH degraded by *Alcanivorax xenomutans* had increased from 0.16 g to 0.28 g indicating an increase of 75 %. Whereas Acinetobacter had increased by 50 % from 0.24 g to 0.36 g on the 30th day. Similarly, the mean TPH degraded had increased by 50 % from 0.28 g to 0.42 g when both bacteria were put together. By the 45th day, the average TPH degraded by Alcanivorax xenomutans had further increased from 0.28 g recorded on the 30th day to 0.38 g, signifying a 36 % increase, a 39 % increase observed in *Acinetobacter Junii* degraded (0.36–0.5) while having the two bacteria together resulted in a 29 % increase (0.42–0.54). For all the three days that the experiment was recorded, *Alcanivorax xenomutans* degraded the least amount of petroleum hydrocarbons on the average for each of the days. Moreover, maximum average TPH degraded was observed on the 45th Day while the least was observed on Day 15.

3.4. Phylogenetic analysis

The neighbor joining approach was employed to deduce the evolutionary history as shown in the optimal tree (Fig. 1). The

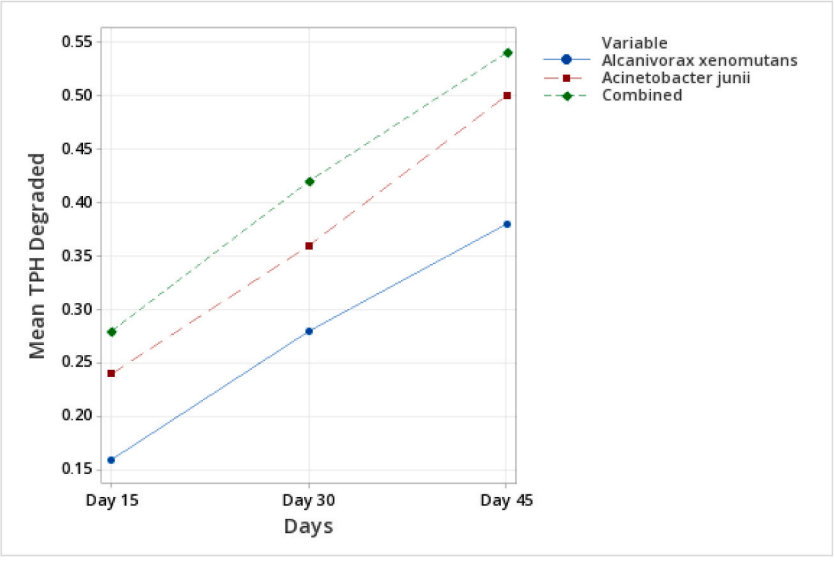

Fig. 1. Average total petroleum hydrocarbon (TPH) degraded by *Alcanivorax xenomutans* and *Acinetobacter junii* singly and in combination over a period of 45 days.

percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The phylogenetic tree was drawn to scale using the same units in the branch lengths as those of the evolutionary distances and used to extrapolate the tree. The Maximum Composite Likelihood technique was also employed to calculate the evolutionary

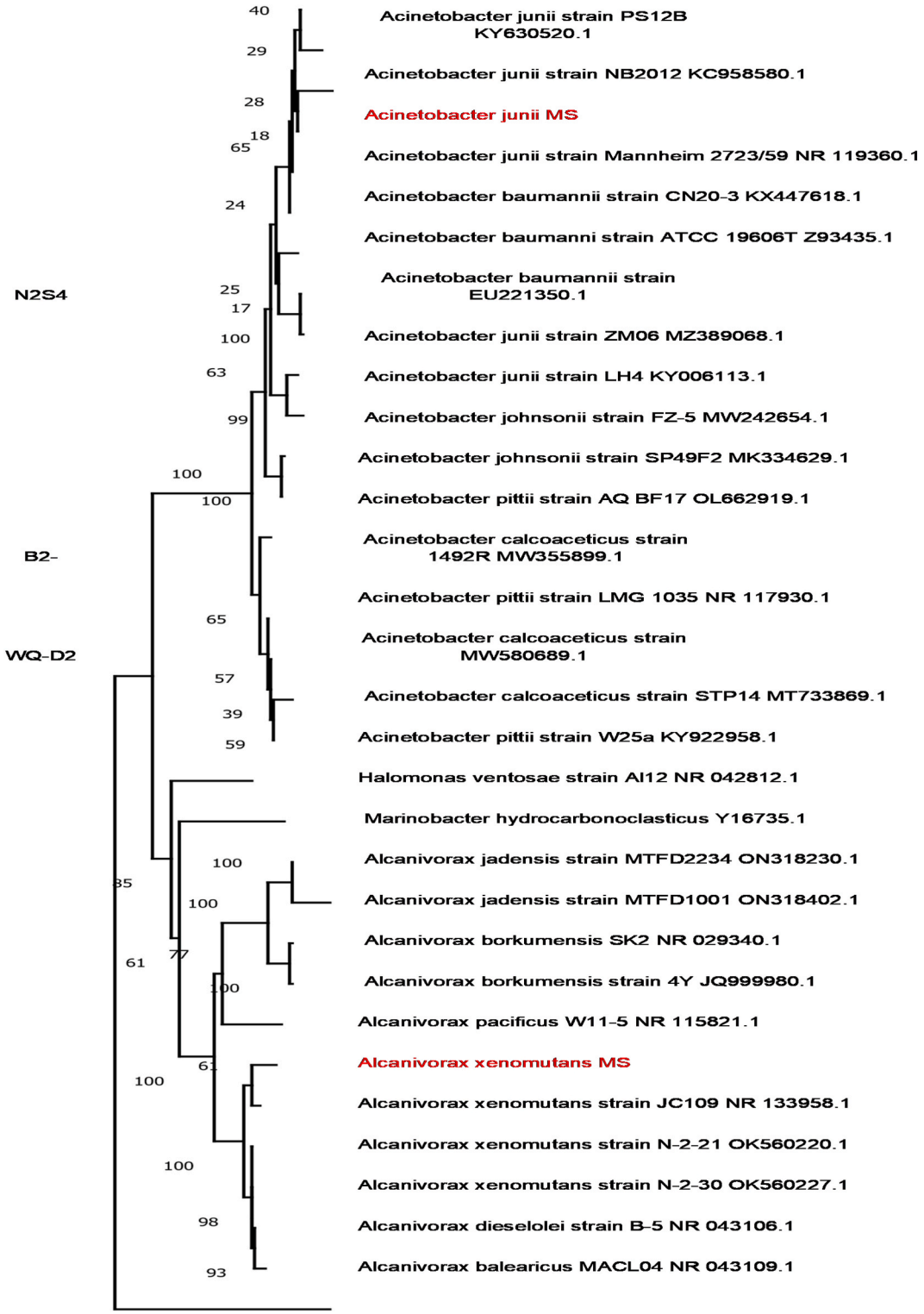

Fig. 2. Phylogenetic tree of *Acinetobacter Junii* and *Alcanivorax xenomutans*.

distances, and were in the units of the number of base substitutions per site. The Ghanaian isolates of *Alcanivorax* and *Acinobacter* clustered with *Alcanivorax xenomutans* and *Acinobacter juni* species respectively in a phylogenetic analysis (Fig. 2). BLAST analysis of sequence data for a 1394-bp fragment of the 16S rRNA gene of the Ghanaian isolate (*Alcanivorax xenomutans* MS) revealed >99 % sequence identity to the range of *Alcanivorax xenomutans* isolates present on the GenBank database. The isolate of closest identity was *Alcanivorax xenomutans* with accession number NR_133958.1 (100 % nucleotide identity). Similarly, the Ghanaian isolate (*Acinobacter juni* MS) shared nucleotide identity of >99.0 % with the *Acinobacter juni* isolates available on the GenBank database, sharing nucleotide identity of 99.93 % with an *Acinobacter juni* isolate with accession number KJ147060.1.

4. Discussion

In vitro microcosm experiments using bacteria species; *Acinetobacter junii* and *Alcanivorax xenomutans*, were successfully undertaken to assess the capacity of degradation of the 2 bacteria species under laboratory conditions. The bacteria were applied alone and, in a consortium, to ascertain the degradation capabilities of the consortium as opposed to the individual bacteria. Cultures containing single microbes may lack the variety of hydrocarbon-degrading mechanisms present within a consortium to effectively undertake crude oil degradation [33]. Furthermore, the power of synergy as opposed to competition, amensalism, mutualism and other forms of relationships when organisms are put together can also improve microbial degradation capacities [34].

This current study utilized MALDI-TOF MS for identification of bacteria isolates after enrichment culture methods, using crude oil as the only source of carbon. A possible genus or species level identification results from MALDI-TOF MS scores >2, and a comparison of the spectra obtained from the present study to the commercial Spectral Archive and Microbial Identification System (SARAMIS) database suggested that the best match for the sample was *Acinetobacter junii*, confirming a species level identification. Similarly, sample two and its duplicate also gave scores >2. This also suggested a genus level or species level identification and its comparison to the SARAMIS also indicated that the best match was *Alcanivorax xenomutans*. MALDI-TOF MS was used for the detection of *Acinetobacter junii* by Yamamuro et al. [35] in a study in which necrotizing fasciitis was found to be caused by *Acinetobacter junii*. In another study, Álvarez-Buylla et al. [36] were successful in determining the usefulness of MALDI-TOF for the identification of Acinetobacter species as compared to other identification methods. Although usage of MALDI-TOF MS as a means of identifying *Alcanivorax xenomutans* is yet to be reported, the bacteria has been connected to several crude oil bioremediation works and has been cultured and characterized using other molecular gene sequencing methods like 16s rRNA technology [37–40].

Heterotrophic bacteria (bacteria that get their food and energy from organic compounds) species; *Acinetobacter junii* and *Alcanivorax xenomutans,* isolated from pristine (no crude oil spillage) sea along the shores of Takoradi and Tema (on the coast of Ghana) were successful for the remediation of crude oil spills in microcosm experiments. At an average salinity and pH values of 32.43 mg L^{-1} and 7.99 respectively, the bacterium with the highest number per milliliter using total plate count at 28 °Cfor a period of 24 h was *Acinetobacter junii* (1.48×10^5 CFU/ml) followed by *Alcanivorax xenomutans* (9.8×10^4 CFU/ml). The levels of salinity and pH values has been reported to affect the number CFU/ml and type of bacteria isolated [41]. Rath et al. [42] has cited the dependence of type and number of bacteria cultured on pH and salinity values, as has Zhang et al. [43]. The number of CFU/ml of bacteria isolates from this study were comparable to the numbers from earlier research [44,45]. Although the bacteria isolated by the other researchers were from crude oil contaminated sites, the similarities in number of CFU/ml may be due to the enrichment isolation methods adopted to culture bacteria in this research. Crude oil was applied as the only source of carbon for the bacteria to utilize, creating an environment similar to a crude oil contaminated site.

Microcosm experiments conducted to ascertain the capacity of degradation showed that *Acinetobacter junii,* when used singly, had appreciable levels of degradation of crude oil regardless of the use of sea water. The first 15 days gave a degradation efficiency of 27.59 %, followed by an efficiency of 41.38 % for 30 days, and an efficiency of 57.47 % for 45 days. Similarly, a halotolerant strain of Acinetobacter used for bioremediation by Qu et al. [46] gave results of an average efficiency of 33.4 % with a half-life period of 6.24–7.79 days, comparable to what was obtained in the current study. Also, Singh et al. [47] revealed the capacity of *Acinetobacter junii* to mineralize toluene (a component of crude oil). Pure *Acinetobacter junii* cultures degraded 73 %, 69 % and 80 % of 100, 150 and 50 ppm toluene concentrations respectively, in a period of three 3 days at 37 °C. The capacity of the *Acinetobacter junii* to mineralize the petroleum hydrocarbons in this research may be attributed to the isolation of the indigenous bacterial species from sea water where it naturally had halotolerance, as well as using crude oil as the only source of carbon during the isolation phase.

Likewise, *Alcanivorax xenomutans* gave comparable degradation efficiencies of 21.14 % at 15 days, 32.18 % at 30 days and 43.68 % at 45 days under parallel microcosm conditions. Similarly, Zadjelovic et al. [38] also showed the capability of A*lcanivorax* sp. to degrade crude oil. However, their work focused on alkanes and some specific genes within the *Alcanivorax* sp. responsible for the biodegradation. Fu et al. [39], also demonstrated the capacity of using culture dependent *Alcanivorax xenomutans* for alkane degradation after 3 days of incubation while Rahul et al. [48] demonstrated the capacity of *Alcanivorax xenomutans* to degrade crude oil.

It was observed however that there were higher rates of degradation in the case of *Acinetobacter junii* for all the three different termination days as opposed to *Alcanivorax xenomutans*. It is also worthy of note that after 45 days of remediation, *Acinetobacter junii* was able to remediate more than half of the added crude oil. Furthermore, both bacteria species, when used individually, showed a consistent and steady increase in degradation of the TPH from 15 to 30–45 days, indicative of their reliability to be used for crude oil remediation. This is despite their isolation from pristine sea water (uncontaminated crude oil areas).

The application of bacteria consortia for the mineralization of petroleum hydrocarbons is worthy of note. The capacity of combined bacteria may present varied situations in their relationships with consequent effects on the amount of TPH degraded at each given time. The combination of *Acinetobacter junii* and *Alcanivorax xenomutans* into a consortium for the microcosm degradation of crude oil yielded 32.18 % at 15 days and rose to 62.07 % at 45 days. The combination of the two bacteria as a consortium for the remediation of

crude oil is yet to be reported. However, they have been reported in combination with other bacteria widely, for the remediation of petroleum hydrocarbons. Ma et al. [49] in their work demonstrated the capacity of bacteria consortia containing *Acinetobacter junii* strain H11S-25 in a combination with *Pseudomonas* sp. strain H11S-28 and *Nitratireductor* sp. strain H11S-31 for the remediation of total alkanes and poly aromatic hydrocarbons (PAHs). Their results demonstrates that *Acinetobacter junii* can be used in combination with other bacteria to achieve high efficiencies during bioremediation. Additionally, Rehman et al. [50] also demonstrated the ability of bacterial consortia containing two strains of *Acinetobacter junii;* strains TYRH47 and LCRH81 in combination with other bacteria for the successful remediation of crude oil contaminated sites. Other research works [51–53] have also stated the use of *Acinetobacter junii* in consortium with other bacteria for petroleum hydrocarbon remediation.

The use of *Alcanivorax xenomutans* in a consortium with other bacteria for the bioremediation of petroleum hydrocarbons in crude oil spills has not been widely reported. However, Santisi et al. [54] used *Alcanivorax* borkumensis in combination with other bacteria for that purpose. This research has therefore presented a unique opportunity to ascertain the effectiveness of using *Alcanivorax xenomutans* in combination with other bacteria for petroleum hydrocarbon degradation. It was evident that the TPH levels degraded increased over time in all the three scenarios: (*Acinetobacter junii* alone, *Alcanivorax xenomutans* alone and a consortium of *Acinetobacter junii* and *Alcanivorax xenomutans*). Chen et al. [55] established that the rate of degradation of TPH in contaminated environments increased over time. They demonstrated that some soil amendments affected the rate of degradation of hydrocarbons significantly over time by orders of magnitude of 2 %, 20 %, 38 % and 95 %. Sarkar et al. [56] also demonstrated that after 30 days of microcosm-based bioremediation, various treatments yielded TPH degradation efficiencies of ~65 %, 36 % and 86 %. Zuzolo et al. [57] also evaluated the remediation potential of some bacteria over a 270-day period and showed that hydrocarbon degradation rate increased after 60 days of incubation with consequent increase in soil enzymatic activities.

Nucleotide sequencing of the 16S rRNA gene of Ghanaian *Alcanivorax xenomutans* and *Acinetobacter Junii* isolates indicates a high degree of similarity, which did not differ greatly from isolates previously described on the GenBank. Knowledge of the diversity of the bacterial agents is essential for the identification of appropriate bacterial isolates representing major strain variants for effective bioremediation. Differences in the ability to degrade hydrocarbon compounds by different strains of bacteria have been reported [58].

5. Conclusion

Acinetobacter junii and *Alcanivorax xenomutans* were successfully used for the remediation of crude oil spills in microcosm experiments after isolation from pristine sea. The combination of the bacteria (*Acinetobacter junii* and *Alcanivorax xenomutans*) into a consortium for crude oil remediation was also successful. They demonstrated their capacity to be used for crude oil spill remediation by achieving 62.07 % and 57.47 % remediation efficiencies in the single and combined forms respectively. For all the 3 terminal days that the experiment was recorded, *Alcanivorax xenomutans* degraded the least amount of petroleum hydrocarbons on average for each of the days. Moreover, maximum average TPH degraded was observed on the 45th Day while the least was observed on Day 15. In summary, *Acinetobacter junii* performed better in terms of the amounts of hydrocarbons degraded over the 45-day period compared to *Alcanivorax xenomutans*. Meanwhile, combining the two bacteria increased the amount of hydrocarbons degraded over the entire period.

Data availability statement

Data will be made available on request.

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Samuel Fosu Gyasi: Investigation, Conceptualization. **Mark Kwasi Sarfo:** Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Conceptualization. **Amos Tiereyangn Kabo-Bah:** Supervision, Data curation, Conceptualization. **Bright Adu:** Supervision, Software, Investigation, Data curation. **Andrew Sarkodie Appiah:** Resources, Methodology, Investigation, Formal analysis. **Yaw Serfor-Armah:** Validation, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that we have no known competing financial interests or personal interests that could have appeared to influence the work reported in this paper.

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