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Degradative Studies of Crude-Oil Degrading Bacteria Isolated from Hydrocarbon Polluted Surface Water in Agbabu, Ondo State

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Authors' contributions

This work was carried out in collaboration between all authors. Authors JOO and TOO designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors PWG and OA managed the analyses of the study. Author TOO managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Hydrocarbon-utilizing potentials of bacteria isolated from twelve different bitumen-contaminated sites in Agbabu, Ondo State were investigated. Water samples were collected and analyzed using standard microbiological techniques. The mean total bacterial count and mean total hydrocarbon-degrading bacterial counts were determined using pour plate technique. The hydrocarbon-utilizing potentials of the isolates were further determined by screening them in mineral salt broth supplemented with 2% crude-oil over a period of 10 days. The growth of the isolates was monitored by measuring the absorbance (OD_{600nm}) and Total viable count (log_{10} CFU/ml). Five isolates selected based on their ability to utilize crude-oil were subjected to conventional biosurfactant screening tests: qualitatively (drop collapse) and quantitatively (oil spreading and emulsification activity). The selected five isolates were used to degrade crude-oil and percentage degradation was determined by Gas chromatography analysis. The isolates were identified by the amplification and sequencing of the 16S rRNA sequences. The Mean Total bacteria count and the Mean Total hydrocarbon-utilizing bacteria count were 7.70 (log_{10} CFU/ml) and 7.14 (log_{10} CFU/ml) respectively.

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Out of the isolates screened for biosurfactant production, only *Pseudomonas* and *Bacillus* species were positive for all the tests. They had a clear zone of 4 mm each and an emulsification capacity of 65.50% and 57.13% respectively. The percentage degradation of crude oil by *Pseudomonas aeruginosa, Micrococcus luteus, Bacillus cereus, Acaligens faecalis* and *B. lichenformis* were 78.67%, 68.27% 63.16%, 54.28% and 51.54% respectively. *Pseudomonas aeruginosa* had the highest % degradation (78.67%) while the lowest % degradation (51.54%) was observed by *B. lichenformis.* The identities of the isolates revealed by 16S rRNA sequences were *Pseudomonas aeruginosa*, *Bacillus cereus*, *B. lichenformis Acaligens faecalis* and *Micrococcus luteus*.

Keywords: Bioremediation; biosurfactant; emulsification index; GC-MS.

1. INTRODUCTION

Crude oil pollution has become a global problem particularly in industrialized and developing countries which results in significant decline in soil quality and chronic sub-acute toxicological effects within ecosystem [1]. Petroleum based fuels are one of the most prevalent pollutants, particularly in developing countries [2]. The widespread nature of petroleum products and their use is strongly associated with anthropogenic discharge of hydrocarbons into the environment [3]. Environmental pollution arising from petroleum leakages in storage tanks, spillage during transportation of petroleum products, deliberate discharge of petroleum products and various industrial processes is hazardous to soil and water ecosystems [4]. This also results in huge disturbances of the abiotic and biotic components of the ecosystem [5]. A major concern for petroleum hydrocarbon pollution is the presence of heavy compounds such as polycyclic aromatic hydrocarbons (PAHs), asphaltenes and many branched compounds with twenty or more carbon atoms [3]. Several approaches such as mechanical and chemical methods have been made to remediate petroleum products from the contaminated area, but these methods generally are expensive and have limited effectiveness. [6]. Bioremediation, defined as the use of microorganisms to detoxify or remove pollutants is regarded as a preferable alternative due to its low cost, high efficiency, environmental friendliness and simplicity technology for long term restoration of crude oil contaminated sites [7,8]

Several species of microorganisms including bacteria, yeasts and fungi obtain both energy and tissue building material from hydrocarbons. A wide range of studies have dealt with biotransformation, biodegradation and bioremediation of petroleum. The ability of many microorganisms in biodegradation of hydrocarbons has been studied by Liangli and Hungchen [9,10,11,4]. These methods are less expensive and do not introduce additional chemicals to the environment.

Hence, this study is aimed at determining hydrocarbon degradation potentials of crude-oil degrading bacteria isolated from bitumen-contaminated surface water.

2. MATERIALS AND METHODS

2.1 Collection of Samples

Surface water samples were collected aseptically from 12 different Bitumen-contaminated sites in Agbabu, Ondo State, Nigeria. All samples were labeled and transported to the laboratory in ice packs for further microbiological analyses.

2.2 Isolation and Screening of Isolates

Hydrocarbon utilizing bacteria were isolated from surface water samples using minimal salt medium (MSM) supplemented with 1% crude oil as a sole source of carbon. The composition of the MSM is as follows: K_2HPO_4 (1.8 g/L); NH_4Cl (4 g/L); $MgSO_4.7H_2O$ (0.2 g/L); NaCl (0.1 g/L); $Na_2SO_4.7H_2O$ (0.01 g/L); agar (20 g/L); carbon source (1%); and distilled water (1L) with pH 7.2. 1ml of each water sample was introduced in 250 ml Erlenmeyer flask containing 100 ml of MSM and 1ml crude oil and incubated at 37°C for seven (7) days. After 7 days, 1.0 ml of sample was transferred into another 250 ml Erlenmeyer flask containing 100 ml fresh MSM and 1% crude oil and further incubated for another 7 days. At the end of 14th day, samples were serially diluted. 0.1 ml from dilutions 10^{-6} and 10^{-7} was spread on MSM agar. The plates were incubated at 37°C for 72h. Pure cultures were obtained using streak techniques and stored at 4°C in agar slant for further use.

2.3 Identification of Isolates

Pure cultures were identified based on their cultural, morphological and biochemical characteristics according to Bergey's Manual of Systematic Bacteriology [12].

2.4 Molecular Identification of the Isolates

2.3.1 Extraction of bacterial DNA

For extracting bacterial genomic DNA, bacterial strain was incubated in 5 ml of medium and bacterial cells were collected from 1 ml of the culture by centrifugation at 13,500 rpm for 3 mins. The pellet was re-suspended in 200 µl of STET buffer (0.1 M NaCl, 10 mM Tris-Cl (pH 8.0), 1 mM EDTA (pH 8.0) and 5% (v/v) Tween 20), from which the genomic DNA was extracted with a guanidium thiocyanate based method (Vuong et al., 2000). Finally the DNA pellet was resuspended in 200 µl of 10 mM Tris-Cl (pH 8.0), 1 mM EDTA (TE) buffer and 5 µl of the extract was run on 1% (w/v) Agarose gel to detect the presence of DNA.

2.3.2 Polymerase chain reaction (PCR)

About 2.5 ng of bacterial genomic DNA was added to a 50 µl PCR mix which contained 1 X Hot start reaction buffer, 0.25 mM dNTPs, 0.01 M (each) primers 27F and 1525R, and 2.5 U Hot start polymerase (Jenabioscience). Thermal cycling was done in a veriti thermal cycler (Applied Biosystems, USA) and cycling conditions were 95ºC for 3 min followed 45ºC cycles of 95ºC for 30 secs, by 45ºC for 1 min, 72ºC for 1 min 30 secs with ramp from 45ºC to 72ºC set at 40%. Subsequently, the reaction was held at 72ºC for 10min after which it was held at 4ºC till terminated. PCR products were resolved on 1% (w/v) agarose gel stained with ethidium bromide and viewed on a transilluminator [13].

2.3.3 Sequencing of amplified 16S rRNA gene

The PCR products were purified using Montage PCR Clean up kit (Millipore). The purified PCR products of approximately 1,500 base pairs were sequenced using primers 27F (5'GAGAGTTTGATCCTGGCTCAG-3') and 1495R (5'-CTACGGCTACCTTGTTACGA-3') (Yang et al., 2015). Sequencing was performed using Big Dye terminator cycle sequencing kit v.3.1 (Applied BioSystems, USA) and sequencing products were resolved on an Applied Biosystems model 3130XL automated DNA sequencing system (Applied BioSystems, USA) at the Macrogen, Inc., Seoul, Korea, [14].

2.4 Screening of Isolates for Biosurfactant Production

Prior to the screening for biosurfactants, the isolates were inoculated into 10ml of MSM each and the incubated at 37°C for 72 h. The culture media were centrifuged at 3000 revolutions per minute (r.p.m.) for 30 minutes. The supernatant was collected and the cells discarded. The supernatant was used for the various biosurfactant screening tests or assays.

2.4.1 Drop collapse assay

The assay was carried out as described by Jain et al. [15]. A drop of the culture supernatant was placed carefully on an oil coated glass slide and observed after one minute. If the drop of supernatant collapsed and spread on the oil coated surface, it signifies the presence of biosurfactant (positive). But if the drop remains after one minute, it was documented as negative. This test was simultaneously carried out on distilled water as control.

2.4.2 Oil spreading assay

Using a micropipette, ten microliter (10 µl) of vegetable oil was added to the surface of 40 ml of distilled water into a petri dish to form a thin oil layer. 10 µl of the culture supernatant was gently dropped on the centre of the oil layer. After one minute, if biosurfactant was present in the supernatant, the oil is displaced and a clear zone was formed as described by Morikawa et al. [16].

2.4.3 Emulsification capacity

Two (2 ml) of kerosene was added to 2 ml of the culture supernatant and the mixture was vortexed at high speed for 2 minutes. The mixture was then incubated for 24 h; the height of the stable emulsion layer was measured. The emulsion index E_{24} was calculated as the ratio of the height of the emulsion layer and the total height of liquid.

$$E_{24} = \frac{h_{emulsification}}{h_{total}} \times 100\%$$

2.5 Extraction of Residual Crude Oil

On the 28th day after readings were taken and recorded, the flasks were shaken vigorously to

suspend the solid materials in order to obtain homogenous sample. The samples were extracted using a separating funnel with a mixture of n- Hexane and concentrate to final volume of 5 ml. A 1 ml aliquot from each sample was separated and analyzed to check the total petroleum hydrocarbon degradation by gas chromatography (GC).

2.6 Gas Chromatography

The extract was analyzed by whole oil-gas chromatography analysis by concentrating the extract to 1.0ml by stream of the nitrogen gas before the gas chromatography analysis. 1-2microliter was injected into the GC column through the injection port. The extraction method for the analysis of hydrocarbons profiles in the samples was by employing the modified methods of ASTM D3328 and ASTM 3415. The oil sample was carefully emptied into a 27 ml capacity McCartney bottle of borosilicate material and 10ml of the ratio 3:1 redistilled hexane: dichloromethane was added. The bottle and its content were placed in the sonicator to extract the hydrocarbons for about 2 hours. The organic layer was filtered into 250 ml capacity borosilicate beaker. The extract was carefully transferred into a 1litre separating funnel, and 20 ml of the redistilled dichloromethane was added. The separator funnel was shaken vigorously for about 2 minutes with periodic venting to release vapour pressure. The organic layer was allowed to separate for 10 minutes and was recovered into the 250 ml flask. The aqueous layer was re-extracted twice with 20 ml of the extractant. The combined extract was dried by passing through the funnel containing the anhydrous sodium sulphate. The dried extract was concentrated with a stream of nitrogen gas.

3. RESULTS AND DISCUSSION

Five potential microbes capable of crude oil degradation were isolated from the surface water samples analysed. The identities of the isolates as revealed by 16S rRNA sequences were *Pseudomonas aeruginosa, Bacillus cereus*, *B. lichenformis, Acaligens faecalis* and *Micrococcus luteus.* The strains of *Pseudomonas*, *Bacillus* and *Micrococcus* have been reported to be isolated from oil polluted soil have been reported to possess the ability to utilize crude oil as a sole source of carbon and energy [17].

This study also shows that two of the selected isolates have the ability to produce biosurfactants. *Bacillus* species and *Pseudomonas* species were both positive for the various biosurfactant tests they were subjected to which ultimately confirms the fact that they are good biosurfactant producers; this is in accordance to Tabatabaee et al. [18] and Jaysree et al. [19]. The two species; *Pseudomonas* and *Bacillus* were positive for the qualitative screening and had a clearing zone of 4 mm each and an emulsification capacity of 65.50 % and 57.13% respectively. *Pseudomonas aeruginosa* produces glycolipids which act as emulsifiers or surface-active agents; consequently reducing the surface tension of hydrophobic molecules and leading to their breakdown thereby enhancing the growth of bacteria and the rate of bioremediation [20,21].

Table 1. Mean total bacterial count and mean total hydrocarbon-degrading bacteria count (Log_{10}cfu/ml) of samples obtained from Agbabu, Ondo State

Samples	MTBC Log_{10}Cfu/ml	MTHDBC Log_{10}Cfu/ml
AGW1	6.25	4.77
AGW2	7.58	5.40
AGW3	7.61	5.35
AGW4	8.04	6.40
AGW5	7.68	6.22
AGW6	7.55	5.35
AGW7	7.58	6.29
AGW8	7.75	4.64
AGW9	8.77	5.20
AGW10	8.26	5.52
AGW11	7.57	4.36
RANGE	6.25-8.77	4.36-6.40
MEAN	7.69	5.41

Table 2. Screening of bacterial isolates for biosurfactant production

Isolates	Heamolysis (mm)	Drop collapse assaY	Oil spreading assay diameter (mm)	Emulsification activity (%)
E. aerogenes	_	-	No reaction	-
Staphylococcus sp.	-	+	2.5	45.33
P. aeruginosa	+	+	4.0	65.50
A. feacalis	-	-	No reaction	-
B. lichenformis	+	+	3.0	51.33
Micrococcus sp	-	+	No reaction	
B. cereus	+	+	4.0	57.13

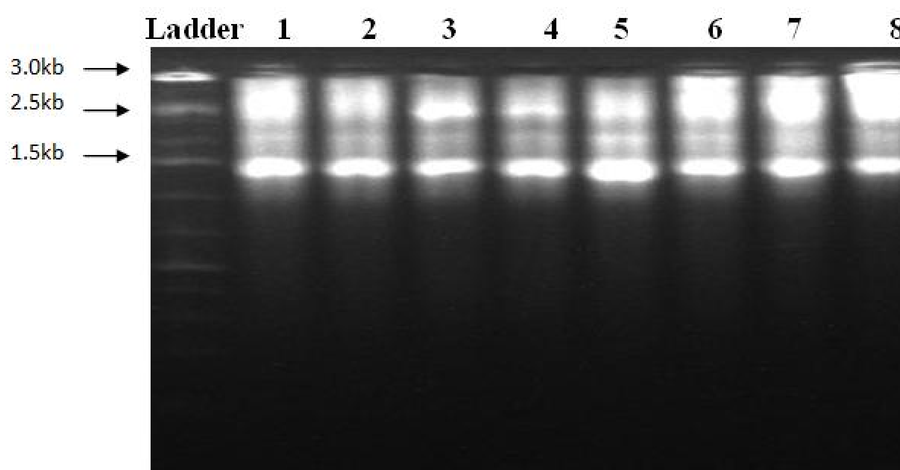

Plate 1. PCR amplification of the bacteria isolates

Plate 2. Tube showing emulsification ability of biosurfactant-producing bacteria

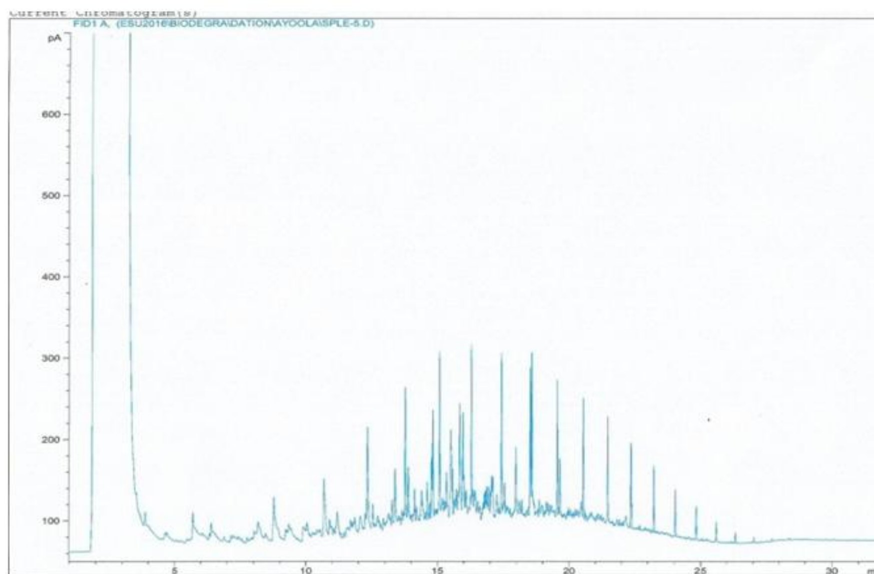

Fig. 1. Gas chromatographic traces of n-hexane extract of residual crude-oil from culture fluids of control at 28 days incubation period

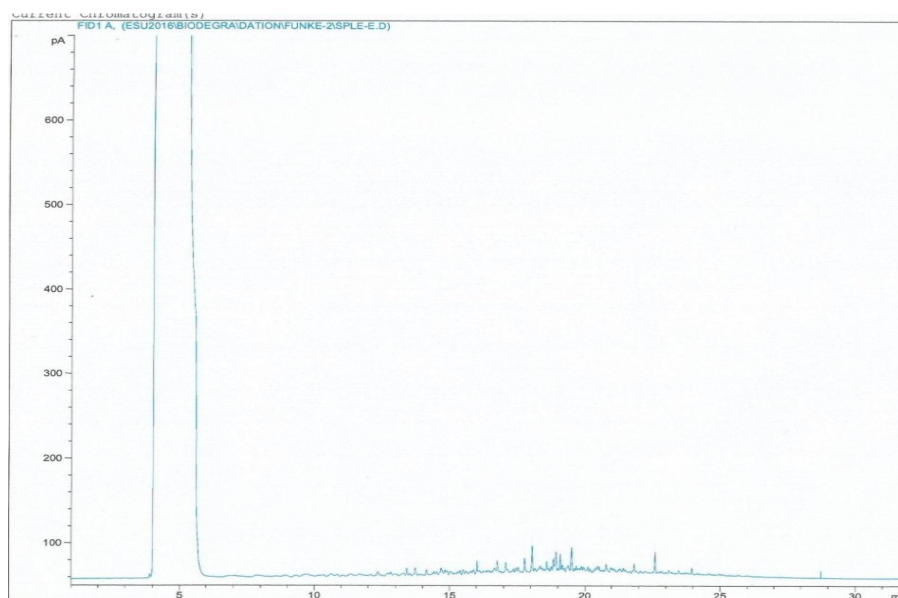

Fig. 2. Gas chromatographic traces of n-hexane extract of residual crude-oil from culture fluids of *Micrococcus luteus at* 28 days incubation period

3.1 Gas Chromatographic Analysis

Analysis of GC results showed that all the microbes are prospective crude oil degraders. They were capable of degrading almost all the hydrocarbons present in the crude oil. Fig. 1 shows the chromatogram of n-hexane extract of Crude-oil. Fig. 2 to Fig. 6 shows chromatographic traces of n-hexane extract of residual Crude-oil from each isolate culture fluids at 28 days incubation period. The residual analysis from each isolate culture fluid compared to that of the control showed that many of the hydrocarbons were degraded in the inoculated samples. The percentage degradation of crude oil by *Pseudomonas aeruginosa, Micrococcus luteus,*

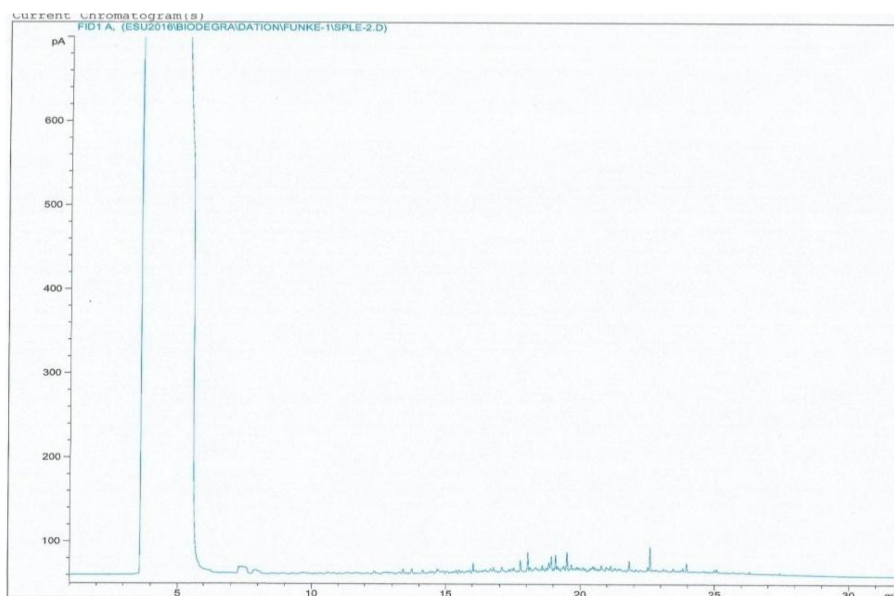

Fig 3. Gas chromatographic traces of n-hexane extract of residual crude-oil from culture fluids of *Pseudomonas aeruginosa at* 28days incubation period

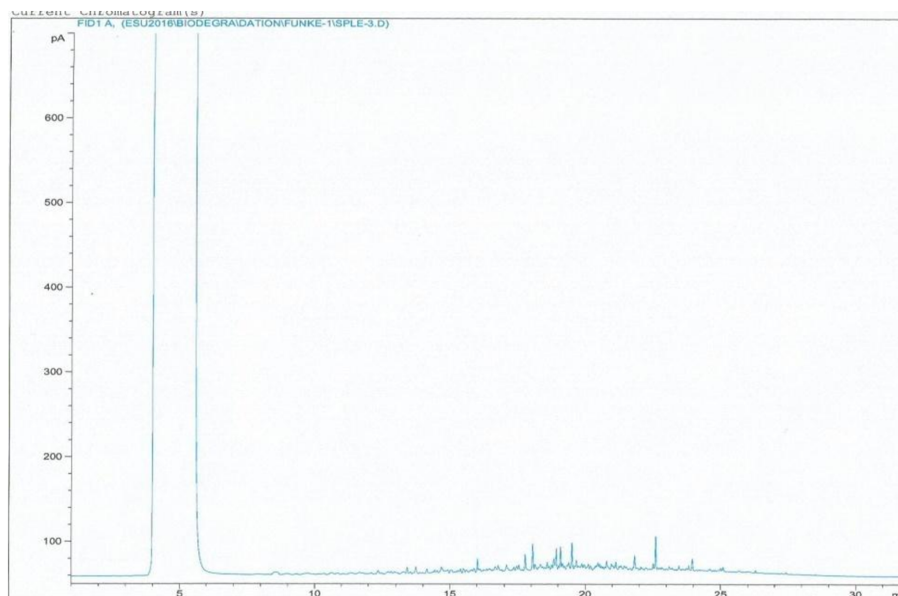

Fig 4. Gas chromatographic traces of n-hexane extract of residual crude-oil from culture fluids of *Bacillus cereus at* 28 days incubation period

Bacillus cereus, Acaligens faecalis and *B. lichenformis* were 78.67%, 68.27% 63.16%, 54.28% and 51.54% respectively. *Pseudomonas aeruginosa* had the highest % degradation (78.67%) while the lowest % degradation (51.54%) was observed by *B. lichenformis.* The ubiquity, catabolic versatility and genetic flexibility of *Pseudomonas* species in hydrocarbon polluted environments have been reported, the genus *Pseudomonas* is reputed to possess broad substrate affinity for different classes of hydrocarbons such as alkanes,

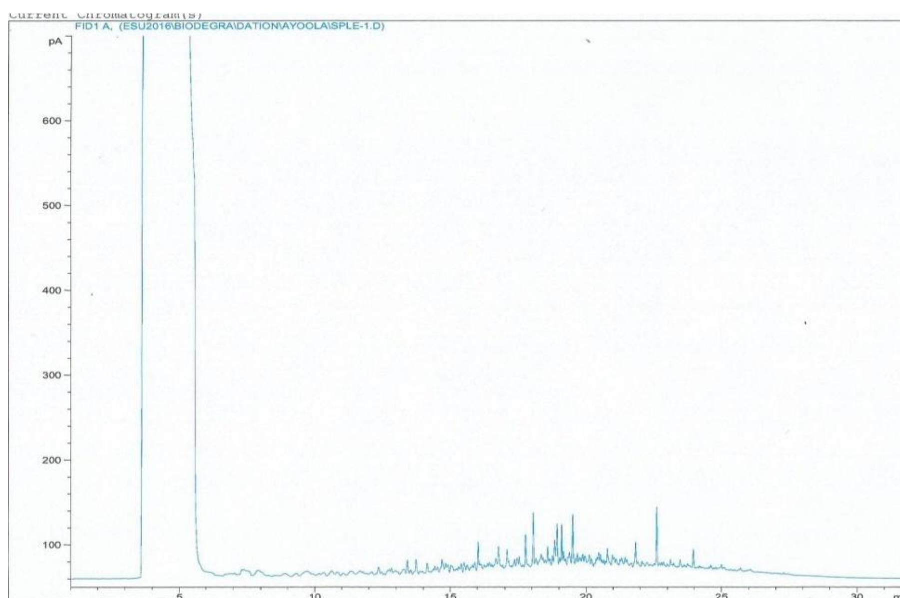

Fig 5. Gas chromatographic traces of n-hexane extract of residual crude-oil from culture fluids of *Acaligens faecalis at* 28days incubation period

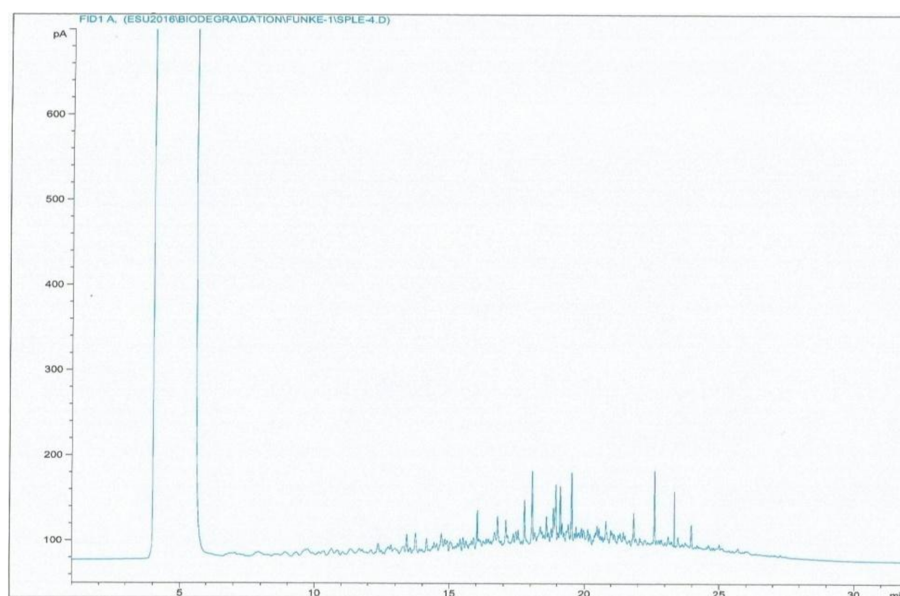

Fig 6. Gas chromatographic traces of n-hexane extract of residual crude-oil from culture fluids of *B.lichenformis at* 28days incubation period

alicyclics, heterocyclics and aromatics [22]. The Total Petroleum Hydrocarbon of the extract of the culture fluids showed variation in degrading ability by the isolates. This could be attributed to different metabolic capacities for the mineralization of the crude oil in the bacteria strains.

4. CONCLUSION

There is a need to develop strains that could be used in bioremediation process of hydrocarbon polluted sites to protect both aquatic and terrestrial environment. The results of this study showed that the isolated bacteria were able to use the hydrocarbon fractions as sole carbon

source. These isolates can be harnessed in an attempt at developing strains that will be useful for the treatment of hydrocarbon-polluted surface water.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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