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Systematic Intrinsic Biodegradation Studies of Crude Oil Contaminated Soil of Bdere Community in South-South, Nigeria

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Abstract

Crude oil pollution is a perennial environmental menace that has bedevilled the South-South ecosystem of Nigeria. This study was aimed at using gas chromatography-mass spectrometry (GC-MS) technique to investigate the biodegradation capabilities of nine bacterial cultures on crude oil residues in Bdere area in South-South, Nigeria. These microorganisms include Pseudomonas aeruginosa, Bacillus subtilis, Bacillus cereus, Micrococcus spp, Pseudomonas putida, Clostridium spp, Bacillus spp, Streptococcus spp, and Serratia spp. The results from the microbial-degraded samples were compared with an abiotic control. The findings reveal that the total petroleum hydrocarbon (TPH) in the microbial-treated samples was significantly attenuated compared to the control, confirming the microrganism's ability to degrade crude oil components. The primary degradation pathway involved biological oxidation of the aliphatic hydrocarbons, transforming them to primary alcohols, aldehydes, and fatty acid derivatives. Degradation was also observed across a wide range of short and long-chain alkanes, aromatic hydrocarbons, and polycyclic aromatic hydrocarbons (PAHs). However, some resistant compounds persisted, and certain degradation products inhibited the rate of further biodegradation. The generation of new metabolites and intermediates confirmed the effective microbial remediation. These findings expand our understanding of microbial degradation of hydrocarbons, offering potential strategies for environmental remediation of oil-contaminated sites.

Keywords: Biodegradation, Crude oil, Microorganism, gas chromatography-mass spectrometry (GC-MS), total petroleum hydrocarbon (TPH)

INTRODUCTION

Biodegradation, the breakdown of substances by microorganisms, has emerged as a promising approach for remediating crude oil-contaminated sites (Van Hamme *et al.*, 2003). Various biological, chemical, and physical factors contribute to the process, influencing the diversity, structure, and activity of microbial communities that catalyze degradation. These factors include the type and concentration of pollutants, nutrient availability, temperature, pH, and soil moisture (Harayama *et al.*, 2004). Crude oil is a complex mixture of hydrocarbons, which range from light volatile compounds to heavy, recalcitrant non-volatile fractions. Some hydrocarbons, such as aliphatic hydrocarbons, can be readily degraded by a wide variety of microorganisms (Megharaj *et al.*, 2011). In contrast, the degradation of aromatic hydrocarbons, particularly polycyclic aromatic hydrocarbons (PAHs), is slower and typically requires specialized microbial communities. This difference can be attributed to the higher chemical stability and lower water solubility of PAHs, which reduce their bioavailability (Van Hamme *et al.*, 2003).

Bioremediation strategies for crude oil-contaminated sites can be broadly classified into two categories: intrinsic and engineered. Intrinsic bioremediation relies on the natural microbial communities and environmental conditions present at the site (Atlas and Hazen, 2011). However, this process can be slow and is typically suited to low to moderate levels of contamination. Engineered bioremediation, on the other hand, involves the deliberate addition of nutrients (biostimulation), microbial inoculants (bioaugmentation), or both to enhance degradation rates (Udousoro *et al.*, 2014; Atlas and Hazen, 2011).

Biostimulation aims to overcome nutrient limitations that often inhibit microbial activity in contaminated soils. Nitrogen and phosphorus are the most commonly added nutrients, as these are often limiting in soil environments.

However, the efficacy of biostimulation is site-specific and depends on factors such as the nature of the hydrocarbons and the indigenous microbial community (Shaibu *et al.*, 2022; Adebayo *et al.*, 2019; Shaibu *et al.*, 2014; Megharaj *et al.*, 2011) while bioaugmentation, involves the addition of selected, often genetically modified, microbial strains known to degrade specific hydrocarbons. However, the success of bioaugmentation is variable, as introduced strains often fail to compete with indigenous microorganisms or adapt to local environmental conditions (Matthew *et al.*, 2019; Bento *et al.*, 2005. Moreover, the utilization of bioaugmentation techniques has encountered significant inquiries around the ecological impact and public acceptance of genetically modified organisms (GMOs) thereby limiting the widespread application of this approach (Tyagi *et al.*, 2011).

The use of microorganisms for bioremediation has been extensively studied, shedding light on the metabolic capabilities and adaptability of various microbial communities in diverse environments. The degradation potential of many bacterial genera like *Pseudomonas*, *Rhodococcus*, and *Alcanivorax* has been demonstrated in numerous studies (Hassanshahian, 2014; Das and Chandran, 2011). Fungi, especially white-rot fungi such as *Phanerochaete chrysosporium*, have also been identified as efficient degraders of recalcitrant compounds like PAHs (Haritash and Kaushik, 2009).

Ongoing research in the field of metagenomics and microbial ecology provides valuable insights into the microbial dynamics and interactions in crude oil-contaminated environments, further refining these bioremediation strategies. Understanding the functional roles and synergistic relationships of different microbial taxa in hydrocarbon degradation can inform the development of more efficient and sustainable remediation strategies (Techtmann and Hazen, 2016).

This study is aimed at providing deep insights into the bacterial biodegradation of heavy crude from Bdere Community located at Gokana Local Government Area in Rivers state, Nigeria.

MATERIALS AND METHODS

Description of study area and sample collection

Soil samples were collected from different sites within Bdere community in Gokana Local Government Area with longitude 04°66.92'N and latitude 07°28.69'E. A 50 g of the oil polluted soil sample was collected from different sampling sites at the depth of 0-15 cm (first layer) and 15-30 cm (second layer) using hand held auger, and core samples using core cylinders. These samples were used to form a composite prior to analysis. Samples were stored in sterile cellophane bags, well labeled and transported immediately for analysis according standard techniques. The uncontaminated soil sample was collected at a crude oil free garden at Rukpokwu, Obiakpor LGA, Rivers State. It was transported immediately to microbiology laboratory, University of Uyo and used as negative control.

Biodegradation Studies

Isolation of Crude Oil Degrading Bacteria

Crude oil-degrading bacteria were isolated from soil samples. The bacteria were cultured aerobically in mineral salt media (MSM) containing specific nutrients and 1% crude oil, following Kazemzadeh *et al.*, 2022 protocol with minor modification. Cultures were shaken at 120 rpm for 3 hours and incubated at 22°C for five days. Bacterial growth was assessed using a UV 2600 spectrophotometer at 600 nm. Selective solid inorganic media (SSIM) were inoculated with 100 µL of culture broth and incubated at 22°C for 10 days to isolate pure colonies, which were then tested for oil degradation capabilities.

Bacterial Selection

Bacteria that thrived on crude oil as their only carbon source were chosen. The three colonies with the fastest growth were further tested and identified using the Biolog Gen III identification system.

Preparation of Inocula

Inocula of 0.1 mL aliquots of four overnight nutrient broth cultures (3 cultures for each strain individually) was washed twice in physiological saline solution (0.87% NaCl, pH 7.2) and suspended in the same to optical density of 0.1 (OD600) (Al-Wasify *et al.*, 2014).

Biodegradation Assays

The bacterial cells from overnight culture at their log phase of growth were transferred to 250 mL conical flasks, each containing 100 mL of sterile mineral salts medium with (0.2% v/v) crude oil (Al-Wasify *et al.*, 2014).The experiment was carried out in duplicate and uninoculated flasks constituted the controls, accounting for abiotic losses. All flasks were incubated at 22 °C for 2 hours determined intervals of time (7, 14, 21, and 28 days). Residual concentrations of crude oil were determined gravimetrically and by gas chromatography.

Gravimetric Analysis

The content of each flask was taken at the end of the incubation period to assess residual concentrations of crude oil. The extraction was carried out by chloroform (3 samples: 1 chloroform). Sample with chloroform was placed in a separating funnel with continuous shaking. Bacterial biomass was estimated after the culture medium was centrifuged at 1500 rpm for 20 minutes in order to separate the biomass (bacterial cells) for each flask at the end of each incubation period. This biomass was washed several times with water then with chloroform to remove residual hydrocarbons. Then it was dried at 100 ℃ to a constant weight (Al-Wasify *et al.*, 2014).

GAS CHROMATOGRAPHY-MASS SPECTROSCOPY ANALYSIS

The samples underwent analysis utilising the Agilent Technologies 7890A Gas Chromatograpy (GC) and 5977B Mass Selective Detector (MSD) using the USEPA EPA 418.1. The experimental conditions of the GC-MS system employed in this study were as follows: The capillary column used in this study is an H 5-MS standard non-polar column with the following specifications: length of 30 m, inner diameter of 0.25 mm, and a film thickness of 0.25 µm. The flow rate of the mobile phase, which consisted of helium as the carrier gas, was established at a rate of 1 ml/min. During the gas chromatography procedure, the oven temperature, was increased from 40˚C to 250˚C at a rate of 5˚C per minute. Additionally, the injection volume used was 1 µl. The experiment involved analyzing samples that were dissolved in methanol. The samples were subjected to a full scan within a mass range of 40-65 m/z. These results were compared with the National Institute of Standards and Technology (NIST) Spectral library (Christie and Han 2010).

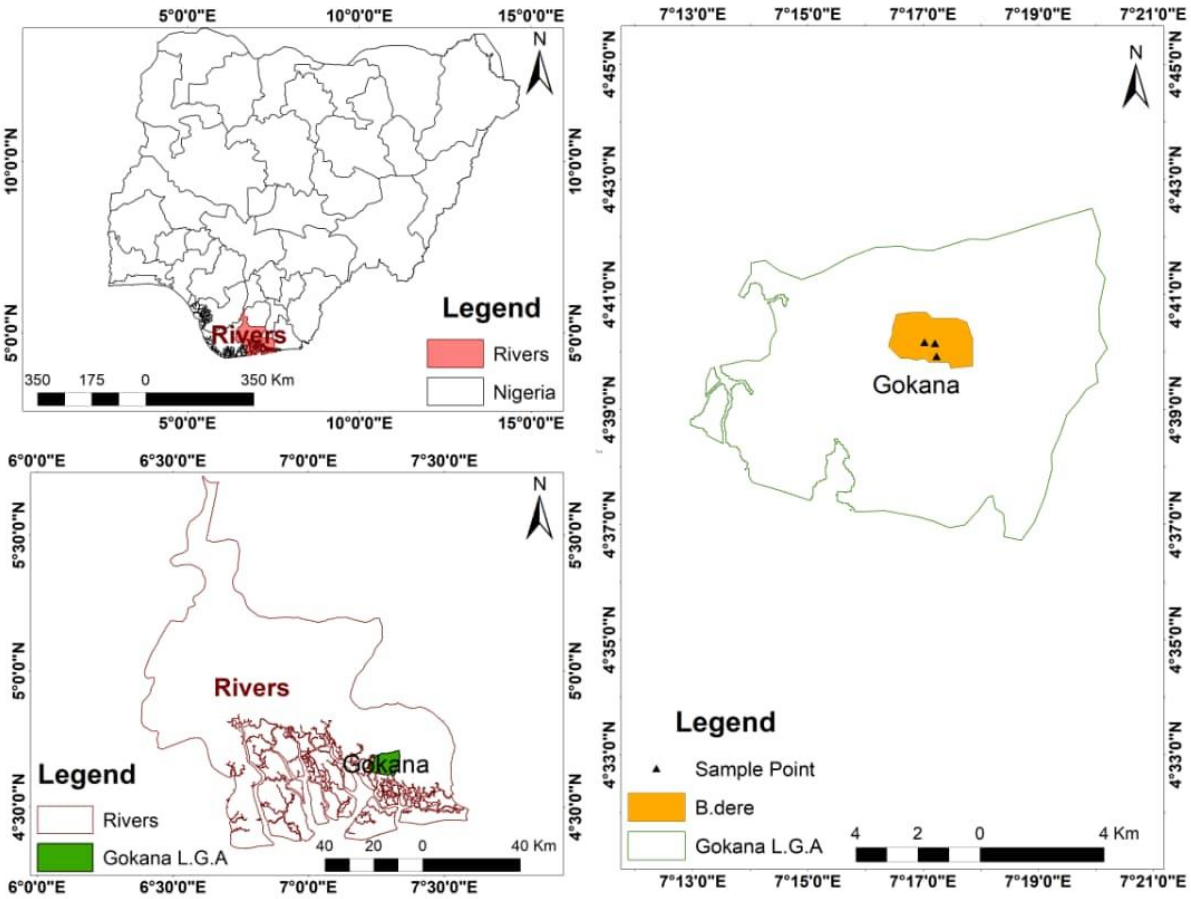


Figure 1: Description of sample site in Bdere in Gokana local government in Rivers state, Nigeria

RESULTS AND DISCUSSION

GC-MS of abiotic control sample of the crude oil

The GC-MS of the control sample (untreated crude oil) (Fig. 2A) along with the chromatograms of the most prominent compounds in terms of abundance is presented (Figs 2B and 2C).

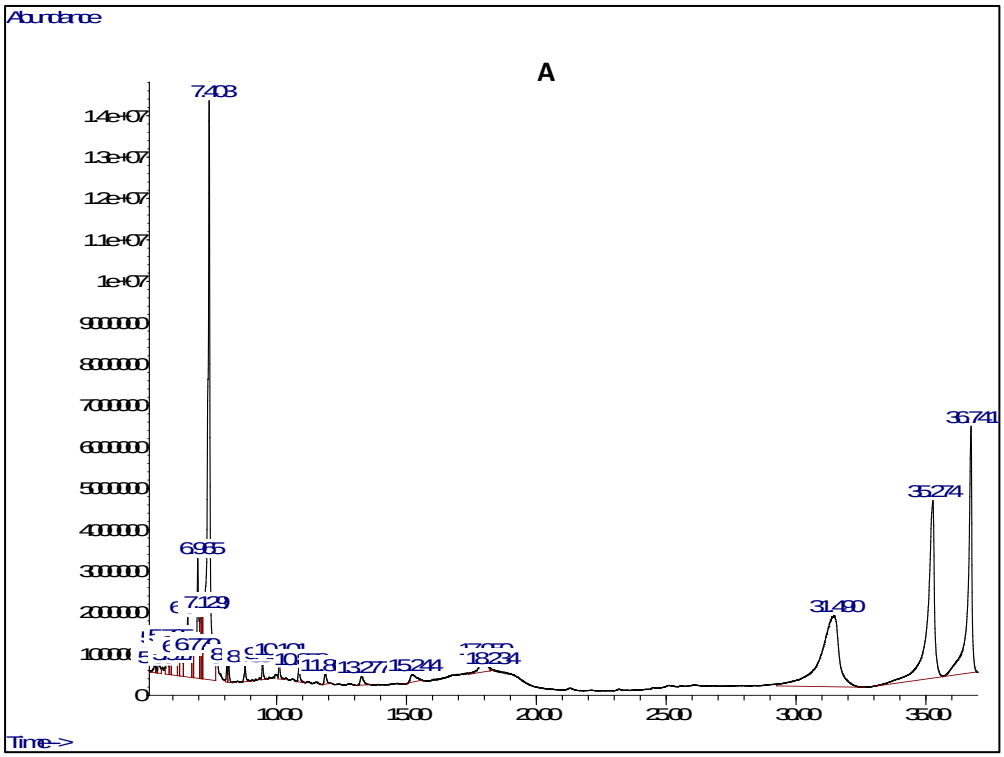
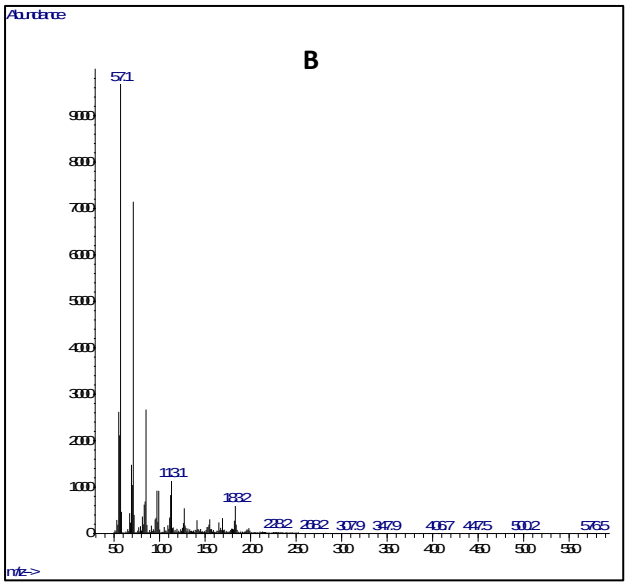
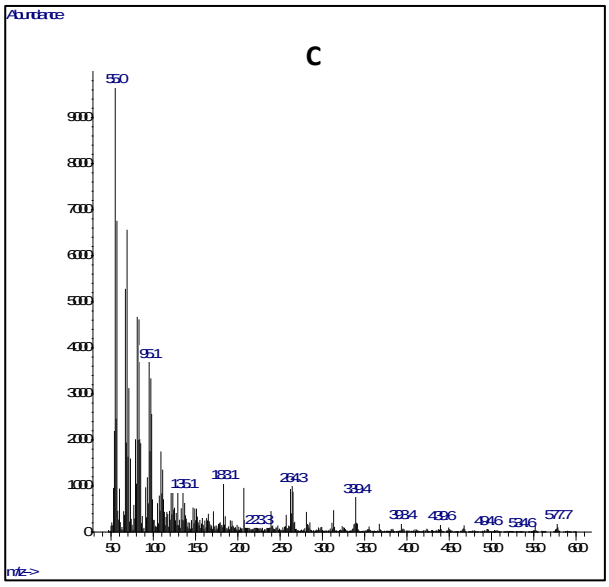


Figure 2A: GC chromatogram of control sample (untreated crude oil), MS chromatograms of (B) heptadecane at RT of 7.403 and (C) 9-Octadecenoic acid at 36.741



GC-MS of bioremediated site of crude oil pollution



GC-MS of bioremediated sample of crude oil pollution with *Pseudomonas aeruginosa*

The GC-MS of bioremediated crude oil with *Pseudomonas aeruginosa* depicting chromatograms of the sample (Fig. 3A) and predominant fractions present at different retention times (Figs. B and C).

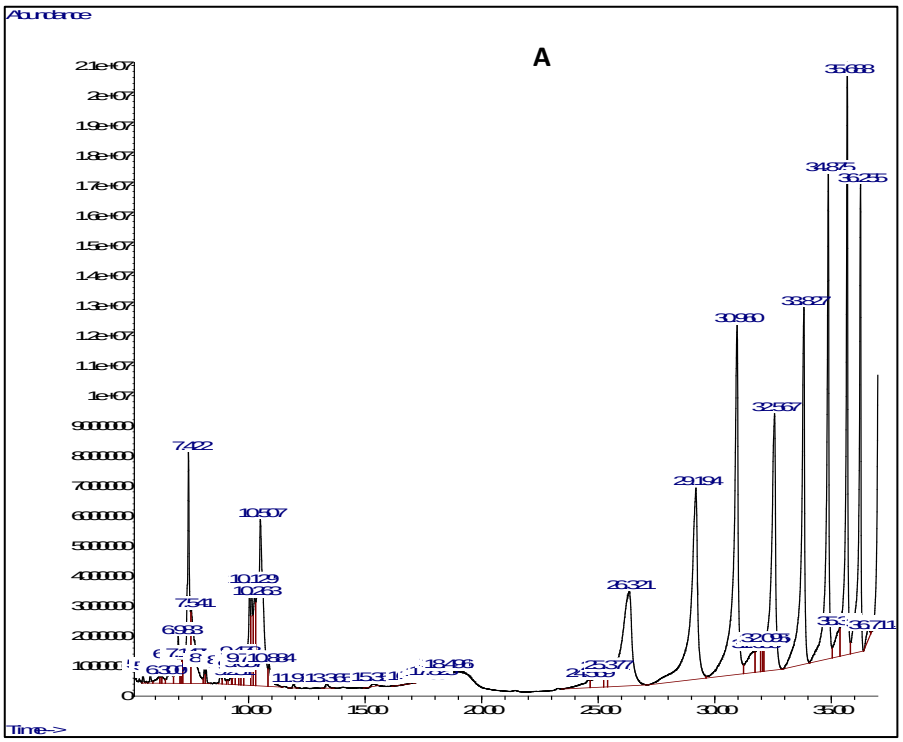
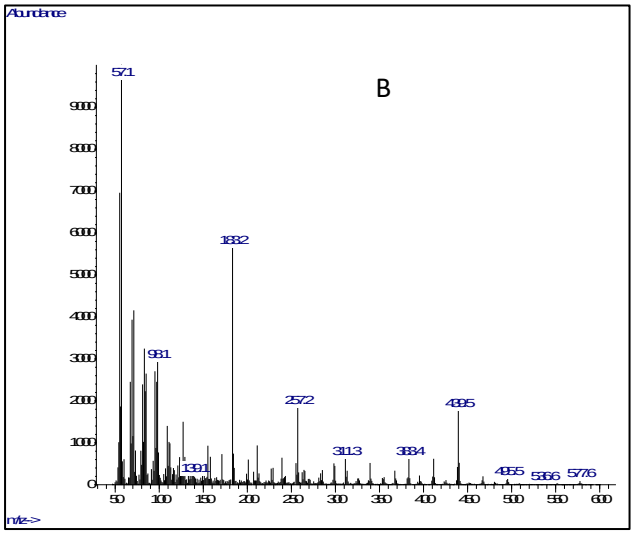
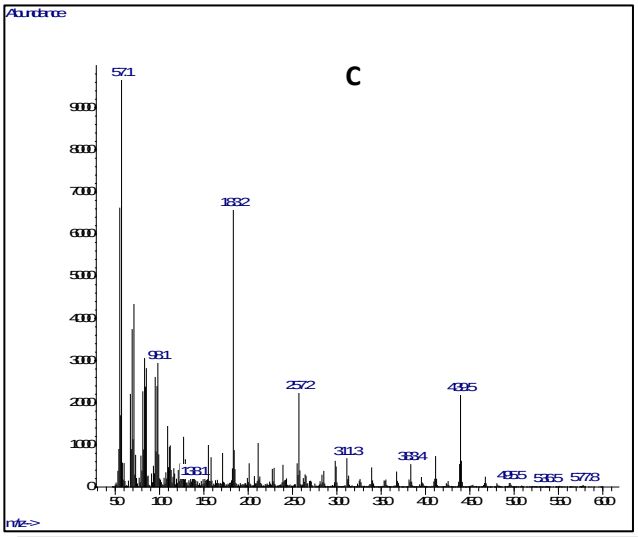


Figure 3A: GC chromatogram of bioremediated sample with *Pseudomonas aeruginosa* MS chromatograms of (B) dodecanoic acid at RT of 35.688 and (C) 1,2,3-propanetriyl ester at 34.875





GC-MS of bioremediated sample of crude oil pollution with *Bacillus subtilis*

The GC-MS of bioremediated crude oil with *Bacillus subtilis* depicting chromatograms of the sample

(Fig. 4A) and predominant fractions present at different retention times (Figs. B and C).

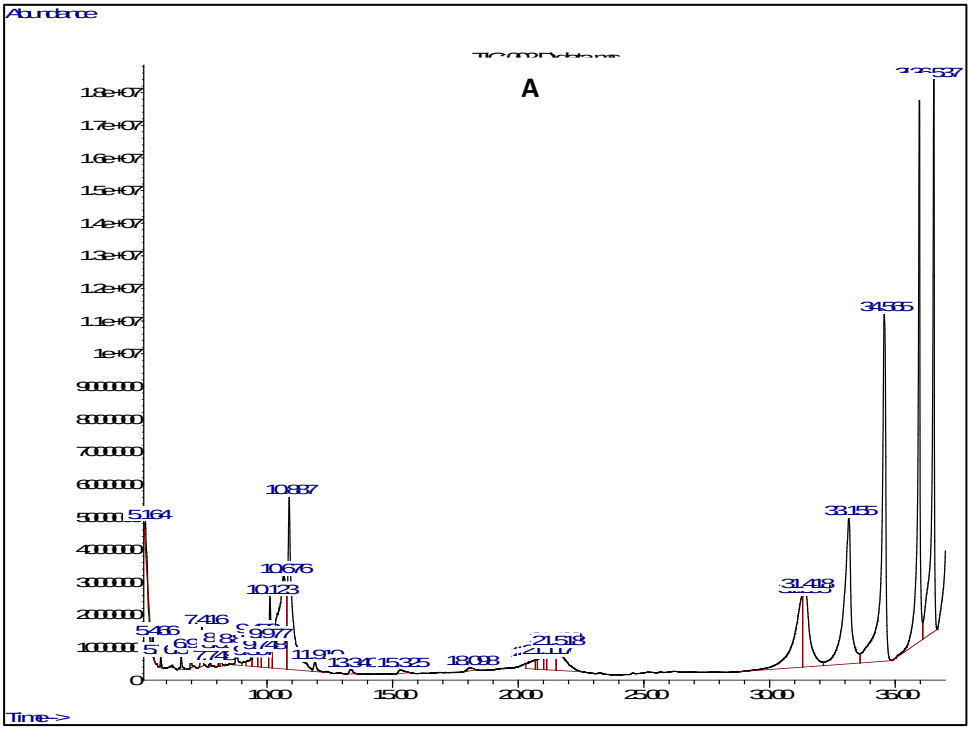
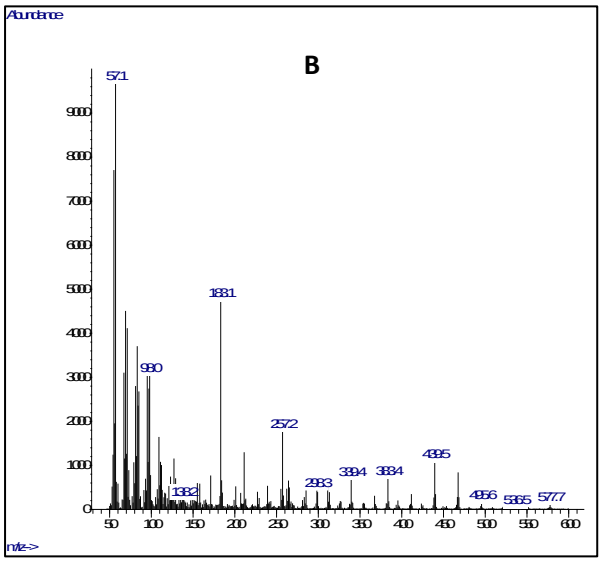
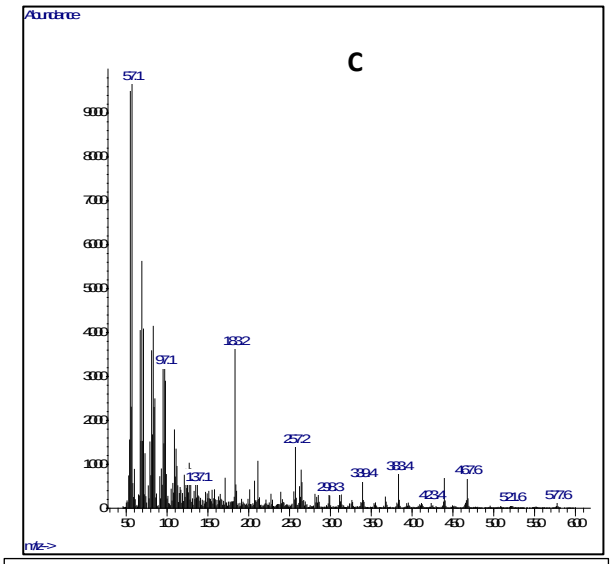


Figure 4A: GC chromatogram of bioremediated sample with *Bacillus subtilis*, MS chromatograms of (B) Methoxyacetic acid at RT of 36.537 and (C) cis-Vaccenic acid at 34.565



GC-MS of bioremediated sample of crude oil pollution with *Bacillus cereus*



The GC-MS of bioremediated crude oil with *Bacillus cereus* depicting chromatograms of

(Fig. 5A) and predominant fractions present at different retention times (Figs. B and C).

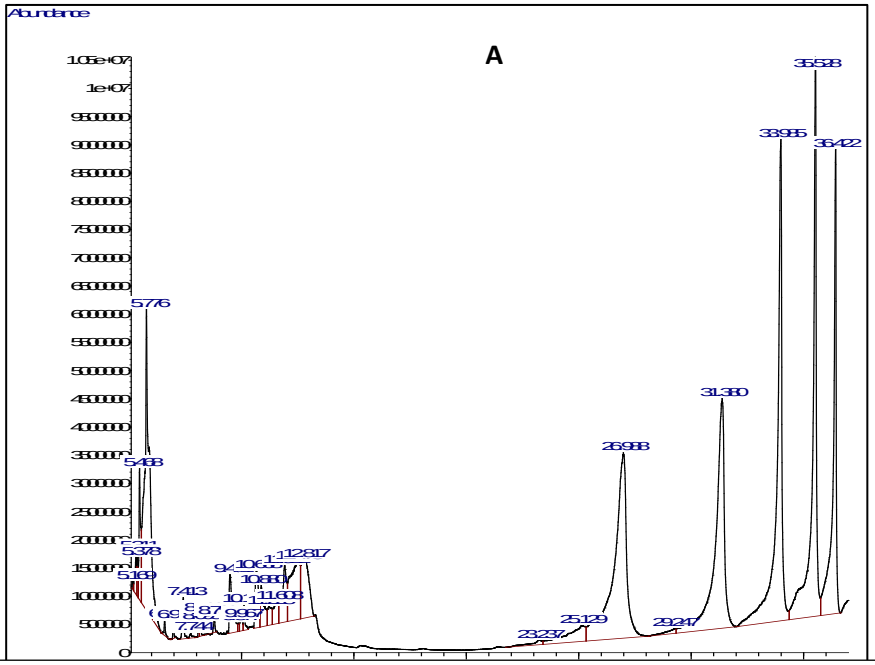
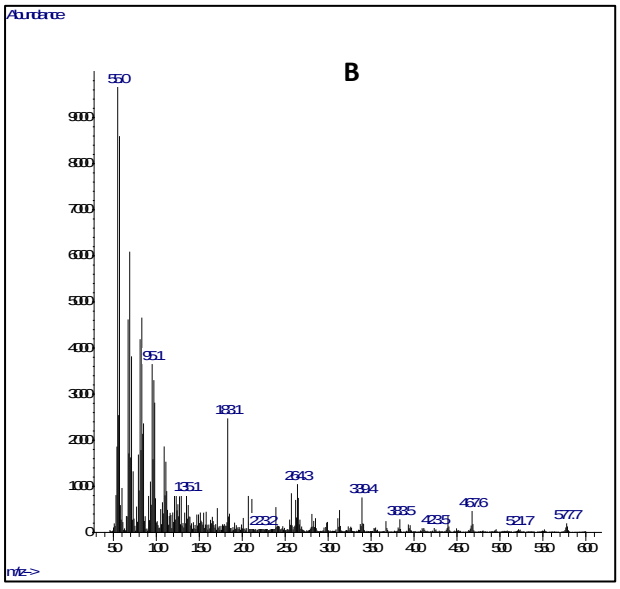
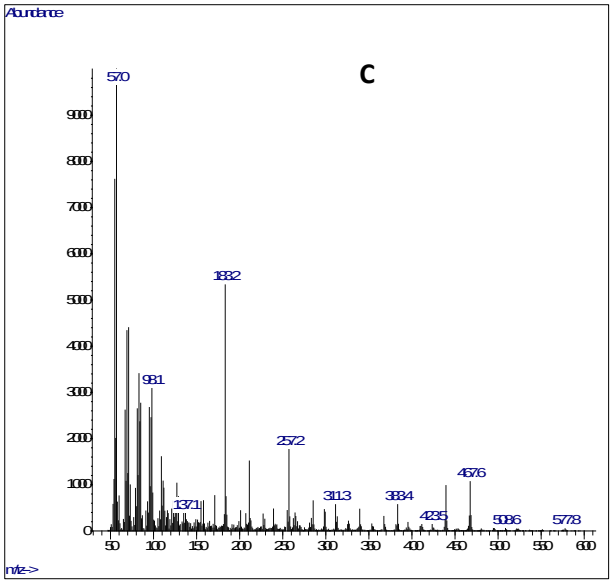


Figure 5A: GC chromatogram of bioremediated sample with *Bacillus cereus*, MS chromatograms of (B) Cyclopentdecanone at RT of 35.528 and (C) dodecanoic acid at RT of 33.985



GC-MS of bioremediated sample of crude oil pollution with *Micrococcus* spp



The GC-MS of bioremediated crude oil from with *Micrococcus* spp depicting chromatograms of the sample

(Fig. 6A) and predominant fractions present at different retention times (Figs. B and C).

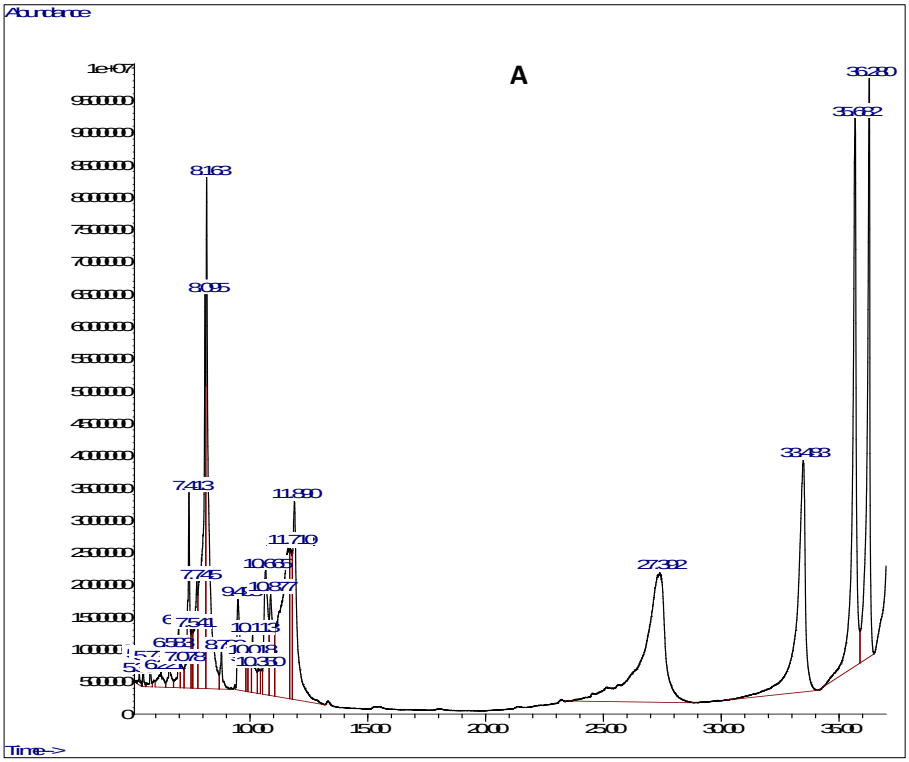
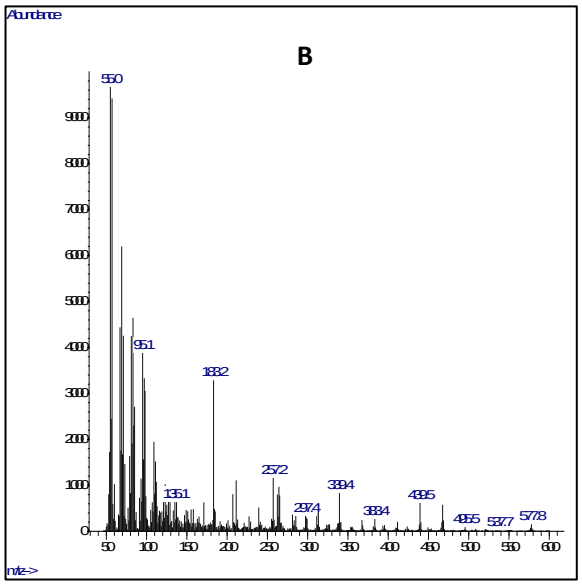
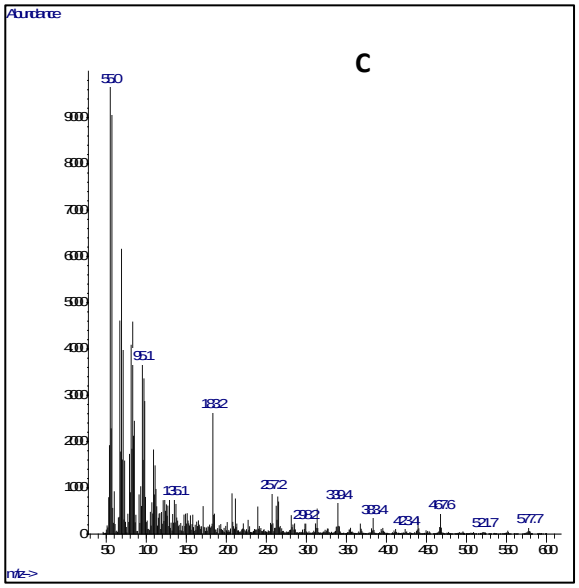


Figure 6A: GC chromatogram of bioremediated sample with *Micrococcus* spp, MS chromatograms of (B) 3Beta-acetoxy-6-nitroandrost-5-en-17-one at RT of 36.27 and (C) 17-Pentatriacontene at RT of 35.682



GC-MS of bioremediated sample of crude oil pollution with *Pseudomonas Putida*



The GC-MS of bioremediated crude oil with *Pseudomonas Putida* depicting chromatograms of the sample

(Fig. 7A) and predominant fractions present at different retention times (Figs. B and C).

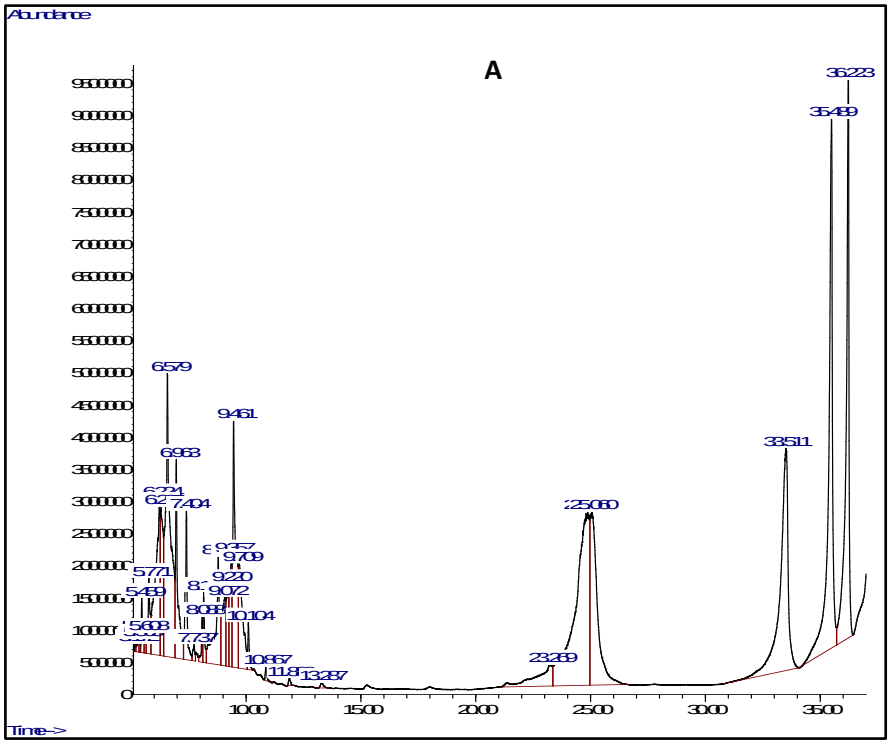
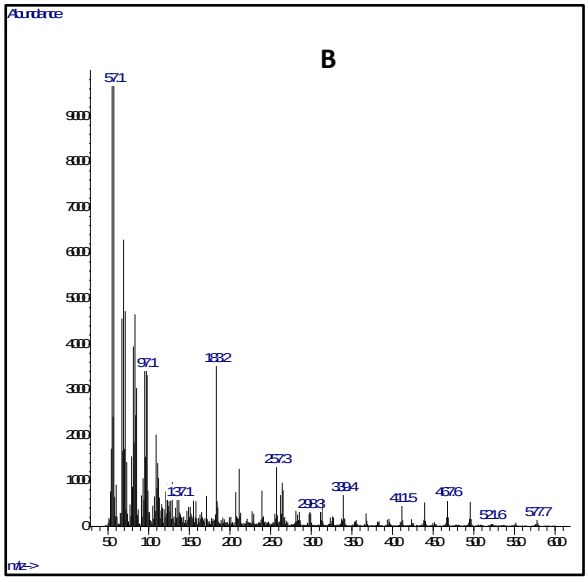
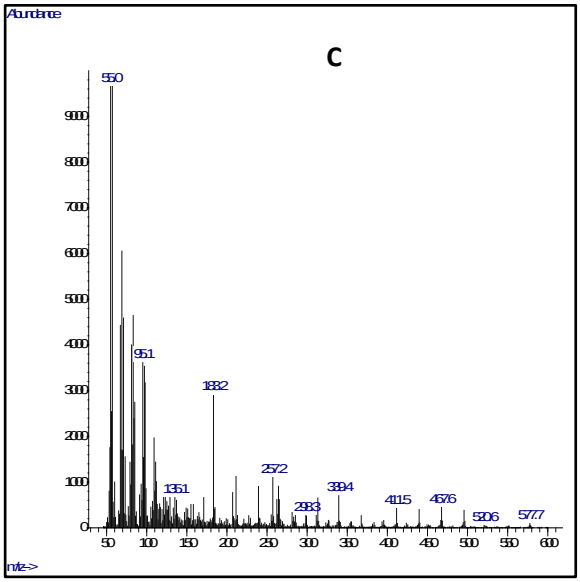


Figure 7A: GC chromatogram of bioremediated sample with *Pseudomonas putida*, MS chromatograms of (B) 9-Octadecenoic acid at RT of 36.22 and (C) 3Beta-acetoxy-6-nitroandrost-5-en-17-one at RT of 35.489



GC-MS of bioremediated sample of crude oil pollution with *Clostridium* spp



The GC-MS of bioremediated crude oil with *Clostridium* spp depicting chromatograms of

(Fig. 8A) and predominant fractions present at different retention times (Figs. B and C).

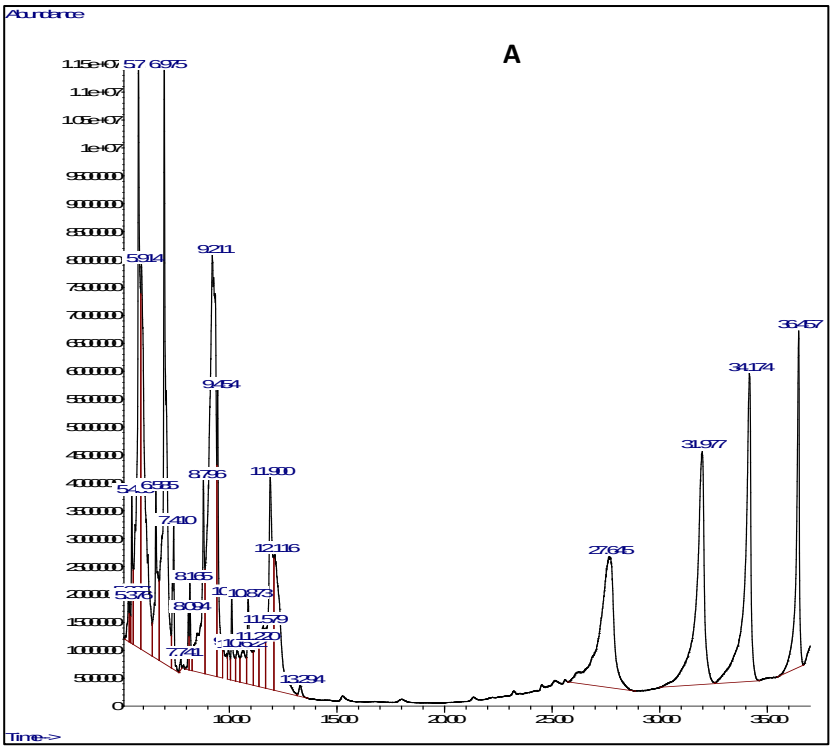
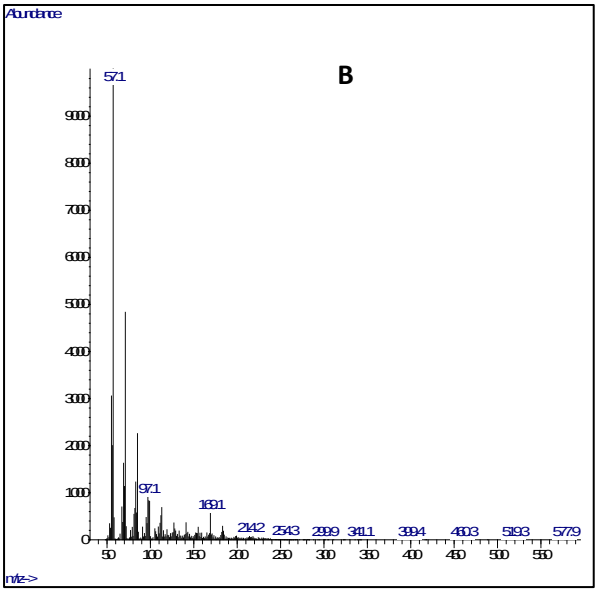
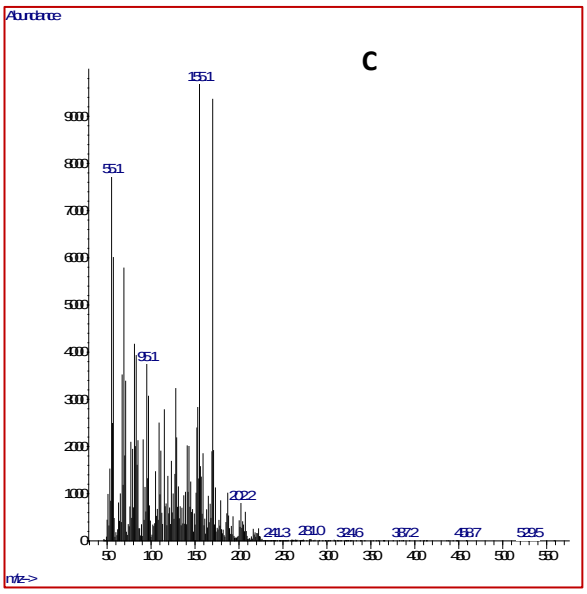


Figure 8A: GC chromatogram of bioremediated sample with *Clostridium* spp, MS chromatograms of (B) Pentadecane at RT of 6.975 and (C) Naphthalene at RT of 5.914





GC-MS of bioremediated sample of crude oil pollution with *Baccillus* spp

The GC-MS of bioremediated crude oil with *Baccillus* spp depicting chromatograms of the sample

(Fig. 9A) and predominant fractions present at different retention times (Figs. B and C).

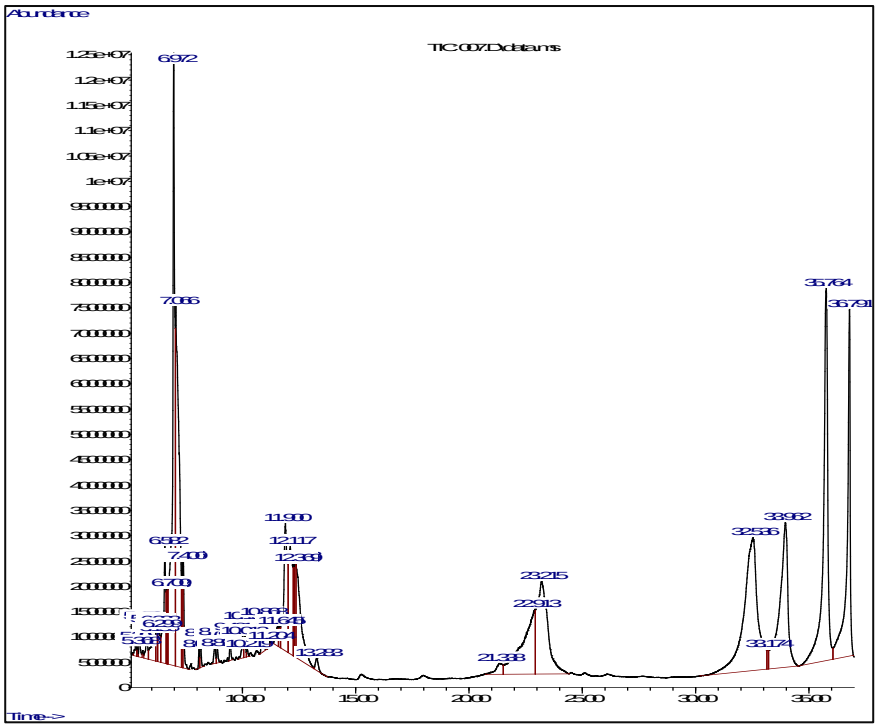
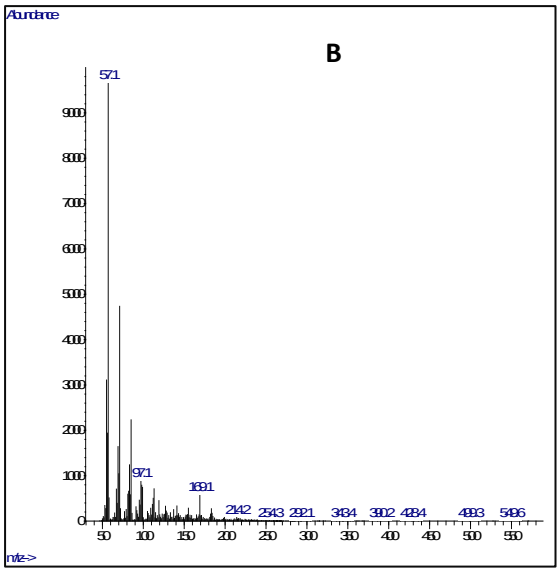
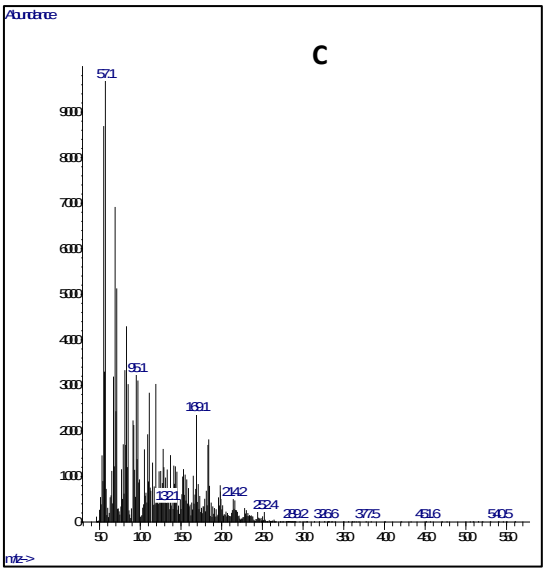


Figure 9A: GC chromatogram of bioremediated sample with *Baccillus* sppspp, MS chromatograms of (B) Pentadecane at RT of 6.972 and (C) Cyclopentane at RT of 7.066





GC-MS of bioremediated sample of crude oil pollution with *Streptococcus* spp

The GC-MS of bioremediated crude oil with *Streptococcus* spp depicting chromatograms of the sample

(Fig. 10A) and predominant fractions present at different retention times (Figs. B and C).

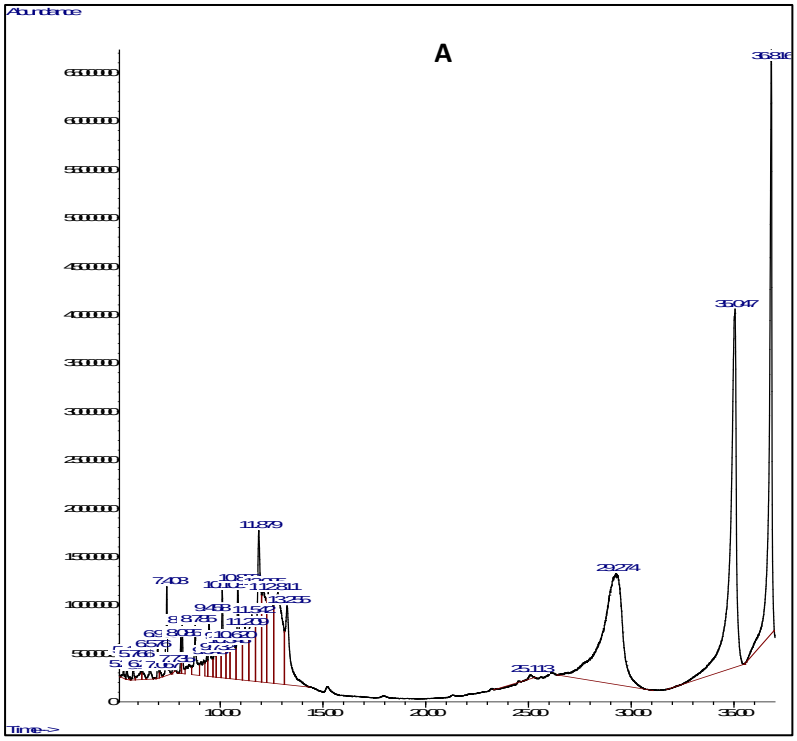
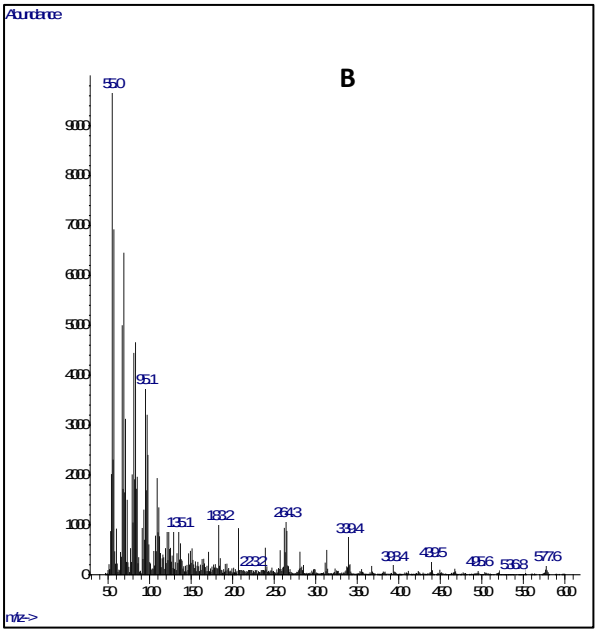
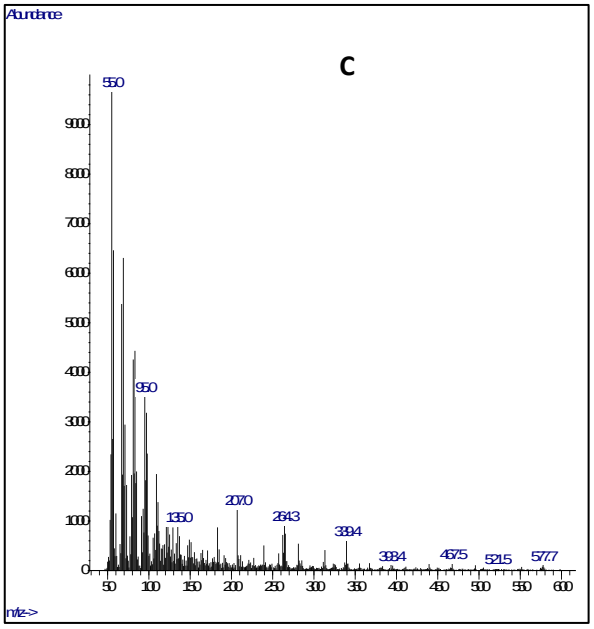


Figure 10A: GC chromatogram of bioremediated sample with *Streptococcus* sppspp, MS chromatograms of (B) 9-Octadecenoic acid (Z)-, 2-hydroxy-1-(hydroxymethyl)ethyl ester at RT of 36.816 and (C) 9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester at RT of 7.066



GC-MS of bioremediated sample of crude oil pollution with *Serratia* spp



The GC-MS of bioremediated crude oil from *Serratia* spp depicting chromatograms of the sample

(Fig. 11A) and predominant fractions present at different retention times (Figs. B and C).

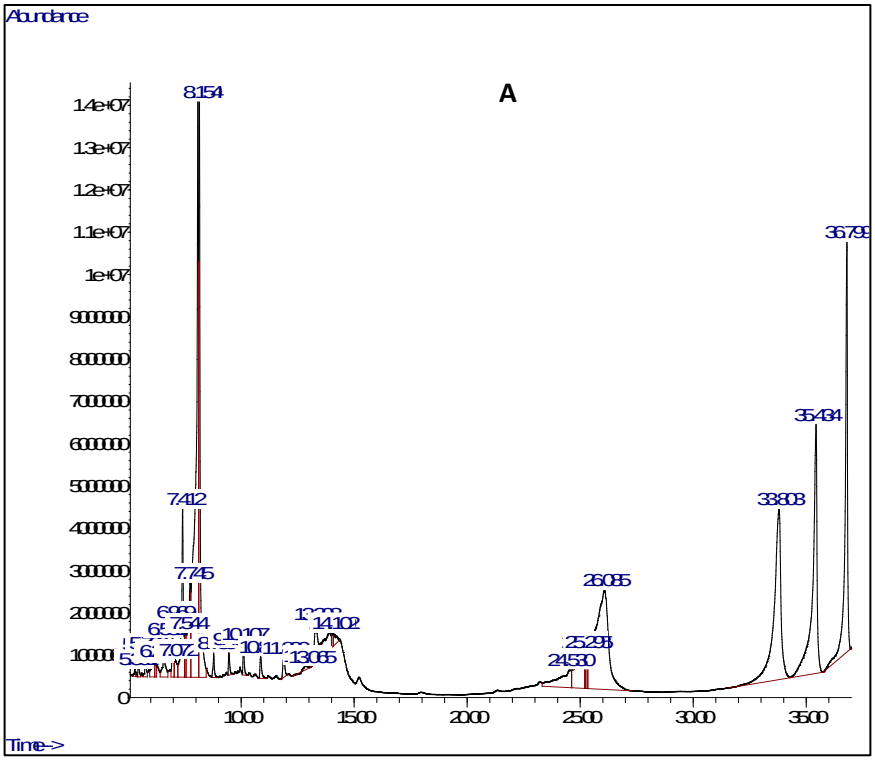
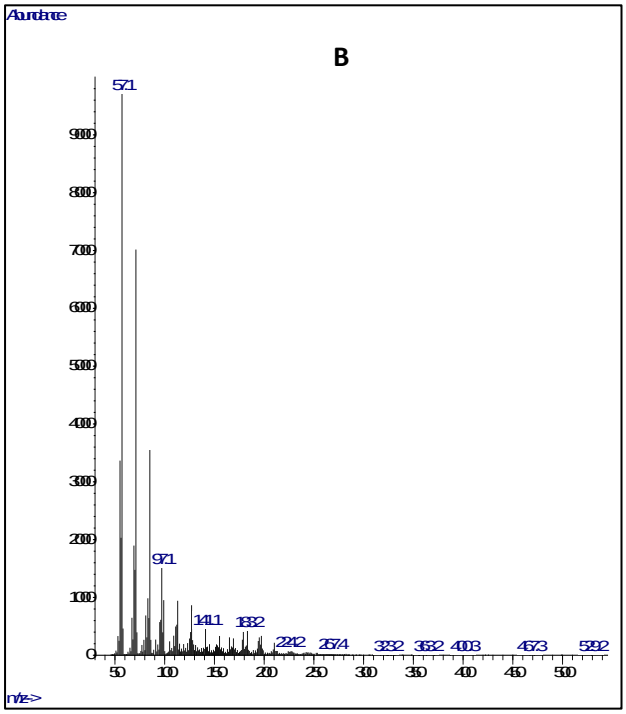
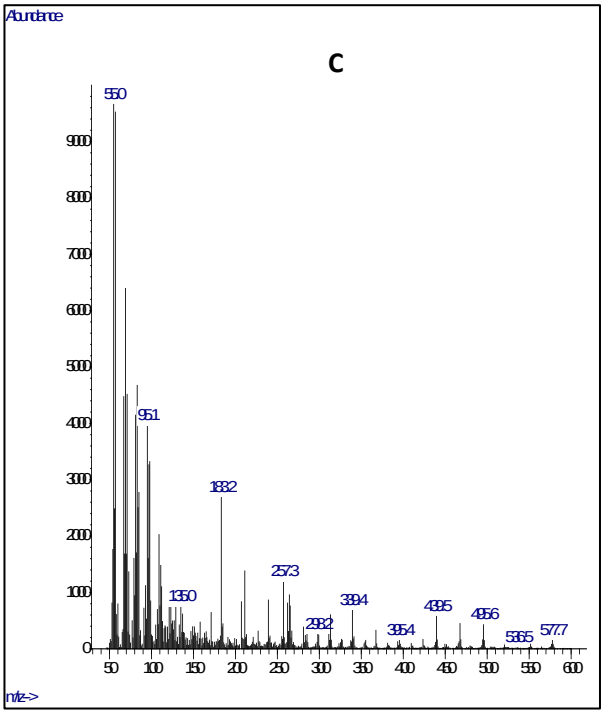


Figure 11A: GC chromatogram of bioremediated sample with *Serratia* spp, MS chromatograms of (B) Hexadecane, 2, 6, 10, 14-tetramethyl-ester at RT of 8.154 and (C) C*is*-7,8-Epoxy-2-methyloctadecane at RT of 36.795





DISCUSSION

Gas chromatography-mass spectrometer analyses of the residual hydrocarbon extracted from 9 different cultures (*Pseudomonas aeruginosa, Bacillus subtilis, Bacillus cereus, Micrococcus* spp, *Pseudomonas putida Clostridium* spp, *Baccillus* spp, *Streptococcus* spp *and Serratia* spp) was compared with an abiotic control sample under the same conditions. The obtained chromatograms are presented in Figures 3 - 119. From the chromatograms, it was revealed that total petroleum hydrocarbon (TPH) is reduced in the sample acted on by the microorganisms compared to the abiotic control assay. This observation confirms the degradative capabilities of the active microorganisms on different crude oil compounds present in the contaminated site.

The microbial activities on the most abundant component in crude oil (aliphatic hydrocarbon) led to degradation initiated via complete biological oxidation of terminal methyl group to a primary alcohol and to the corresponding aldehyde, eventually to the fatty acid derivatives. However, there are situations where the biological oxidation process yielded ω-hydroxy fatty acids from reaction of the terminal side of alkane molecule which eventually got converted to dicarboxylic acids by ß-oxidation (Coon, 2005). According Singh *et al.*, 2012, the secondary alcohol produced from the terminal oxidation of n-alkanes results in the formation of corresponding ketone which is further oxidized by Baeyer–Villiger monooxygenase to an ester that undergoes hydrolyzation by enzyme esterase to an alcohol and a fatty acid. Wide ranges of alkanes (C_{10} to C_{44}) including both short chain and long chain alkanes namely, n-decane (C_{10}) n-undecane (C_{11}), dodecane (C_{12}), n-tridecane (C_{13}), n-pentadecane (C_{15}), n-hexadecane (C_{16}), heptadecane (C_{17}), n-nonadecane (C_{19}), heneicosane (C_{21}), n-docosane (C_{22}), n-tricosane (C_{23}) n-tetracosane (C_{24}), n-heptacosane (C_{27}), hexatriacontane (C_{36}), tetratetracontane (C_{44}) that were present in the abiotic control sample were degraded to their derivatives or the concentration attenuated or complete degradation by the bacterial community. There was also the presence of other aromatic hydrocarbons (1H-1,3-Benzimidazole, p-Benzoquinone, 2-methoxy-5-(methylthio)- and Citronellol including a polycyclic aromatic hydrocarbon (naphthalene) PAH in the control sample (untreated crude oil) which is similar to the observations reported by Al-Wasify *et al.*, 2014 and Arulazhagan and Vasudevan 2009. The individual microorganisms were able to completely degrade the aromatic compounds and the PAH or reduce the concentration of naphthalene in some sample sites due to its recalcitrant nature. Its derivatives were also attenuated to significantly lower concentration after the action of the microbes. This is supported by Arulazhagan and Vasudevan (2009), that reported the degradation of PAHs molecules (phenanthrene and fluorene) at different concentrations to about 95% by halophilic bacterial consortium but this was not the case with *Clostridium* spp where a significant concentration of straight chain alkane (pentadecane) and naphthalene were present. In another study, about 90% degradation of phenanthrene by a bacterial consortium isolated from mangrove sediments was reported by Guo *et al.*, (2005) and Subramaniam *et al.*, (2012) though the problem of accumulation of toxic daughter products of the degradation process which significantly inhibit the consortia activity and rate of degradation still exist (Vidali, 2001).

As shown in the different GC-MS chromatograms of the remediated samples (Figs. 3 – 11), low concentrations of some compounds and several degradation intermediates were found that majorly included organic acids and esters in the bioremediated samples. In remediated sample 1 (Figure 3), all the aliphatic alkanes were degraded to several carboxylic acid at a significant lower concentrations (methoxyacetic acid, cis-13-octadecenoic acid, carbonic acid, propionic acid, oxiraneundecanoic acid, 9-eicosenoic acid, (Z), cis-vaccenic acid, 3-methyl-4-(methoxycarbonyl)hexa-2 ,4-dienoic acid, cis-10-nonadecenoic acid , erucic acid, Z-8-methyl-9-tetradecenoic acid, hexadecanoic acid, Z-8-methyl-9-tetradecenoic acid etc.). Other alcohol, aldehydes and ether compounds present in the treated samples were: 12-methyl-E,E-2,13-octadecadien-1-ol, 2-methyl-Z,Z-3,13-octadecadienol, tert-hexadecanethiol, 7,11-hexadecadienal, 1,14-docosanediol, 13-octadecenal, (Z)-, aspidospermidin-17-ol, Z-2-tridecen-1-ol, methyl 7,9-tridecadienyl ether. Also, some of the ester byproducts detected were octacosyltrifluoroacetate, prop-1-en-2-yltetradecyl ester,tetratriacontyl pentafluoropropionate, ethenyl ester, 1,2,3-propanetriy ester, 4-nitrophenyl laurate, tetradecyl ester, methyl ester, glycidyl ester. There was also the detection of an elevated level of dodecanoic acid and 1,2,3-propanetriyl ester in sample 1 at a retention time of 35.86 which is the predominant product of the biodegradation process in sample 1 by the microbe. Generally, this was the common trend in all the remediated samples (Figs 3 – 11) where discovery of several derivatives was equally observed with absence or reduced concentration of PAH evident.

Marked decrease in the concentrations of compounds previously present in the untreated crude oil (Figure 2) was noticed in the bioremediated samples (Figures3 – 11. The appearance of new peaks resulted either from the degradation of the compounds or the synthesis of new metabolites and intermediates in the biodegradation process (Seo *et al.*, 2009; Singh *et al.*, 2012; Enin *et al.*, 2021).In a crude oil degradation study by Malik and Ahmed (2012) the amount of anthracene and pyrene were depleted to 55.3 and 46.17%, respectively, after treatment by bacterial consortium. In the treated sample some new peaks were observed showing the generation 14 prominent degradation intermediates forming different esters and acids. This report confirms the observations in this study from the chromatograms (Figs. 3 – 11).

CONCLUSION

The biodegradation analysis of the residual hydrocarbons from the of nine bacterial cultures demonstrated significant reduction of total petroleum hydrocarbon (TPH) compared to the abiotic control sample revealing of the considerable potential these microorganisms hold for the bioremediation of crude oil pollutants. The microorganisms effectively degraded a broad range of aliphatic and aromatic hydrocarbons, including naphthalene, which is known for its resistance to degradation. The presence of certain hydrocarbon residues in the treated samples suggests the need for further optimization of the process to increase the efficacy of degradation. The detection of several metabolites and degradation intermediates (methoxyacetic acid, cis-13-octadecenoic acid, carbonic acid, propionic acid, oxiraneundecanoic acid, 9-eicosenoic acid, (Z), cis-vaccenic acid, 3-methyl-4-(methoxycarbonyl)hexa-2 ,4-dienoic acid, cis-10-nonadecenoic acid , erucic acid, Z-8-methyl-9-tetradecenoic acid, hexadecanoic acid, Z-8-methyl-9-tetradecenoic acid) in the remediated samples is indicative of active biological oxidation, yielding various carboxylic acids, aldehydes, alcohols, and esters. The microorganisms' ability to significantly degrade or attenuate a wide range of alkanes, aromatic compounds, and PAHs demonstrates its potential as a promising tool for the remediation of hydrocarbon-contaminated environments.

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