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Detection of Methane-Oxidizing Bacteria and their Use in Petroleum Hydrocarbon Removal

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ABSTRACT

Methylotrophic bacteria have been identified as using carbon one (C1) compounds such as methane. In recent decades, the priority has been on determining new genera of bacteria that have the capability to consume methane and mitigate the effects of global warming. In order to isolate methane-oxidizing bacteria, oil polluted soil samples were collected from Mushrif gas station in Basrah Governorate/southern Iraq. The analysis of *16srRNA* genes identified *Paenibacillus* sp. and *Paenibacillus lautus*, which were able to consume methane as its sole carbon source in NMS medium, also detect the *pMMO* gene (encoded methane monooxygenase enzyme) by real-time PCR. AS far as we know, *P. lautus* was found for the first time to consume methane as a sole carbon and energy source. These bacteria were able to exploite crude oil as their sole source of carbon and energy, recorded the highest growth for *Paenibacillus* sp and *P. lautus* at 1.9 and 0.5 (OD600) after 5 days of incubation successively. The results of gas chromatography analysis revealed high degradation of these bacteria for aliphatic compounds, reaching 92.8% and 89.41% for *Paenibacillus* sp. and *P. lautus*, respectively. Additionally, they exhibited high rates of degradation of aromatic compounds at 98.8% and 97.28%, respectively.

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INTRODUCTION

Common environmental contaminants like petroleum hydrocarbons damage ecosystems (Ashok et al., 2019; Wu et al., 2023). According to certain research, many plants, including those that are edible can absorb toxic petroleum hydrocarbons from polluted soil and aquatic environments (Varjani, 2017; Wang et al., 2023). In terms of total petroleum hydrocarbons, aliphatic and aromatic fractions make up the majority of the molecules (Khan et al., 2018). Accidental spills and leaks during transit or storage are the most typical ways they are introduced into the environment. Total hydrocarbons are mostly deposited in the upper layers of soil when seepage occurs, altering pH, nutrient a viability and biodiversity (Varjani, 2017; Devatha et al., 2019; Martínez-Cuesta et al., 2023).

Petroleum hydrocarbons are the most frequent dangerous contaminants in polluted soils (Haleyur et al., 2019). Some of them as a result of their biodegradable nature can be eliminated by bioremediation. This is done through the activity of a native microbial in the site or may be by introduced microorganisms from and outside source (Di Marcantonio et al., 2023). Numerous hydrocarbon compounds spilled into the environment are decomposed or taken up naturally by microorganisms, which utilize them to meet their carbon demands, requirements for reproduction, and to lessen the physiological stress brought on by oil pollutants (Žvirgždas

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et al., 2023). The biological procedures are regarded as more cost-effective and ecologically benign than the physicochemical and thermal technologies while yet guaranteeing excellent remediation efficiency (Ossai et al., 2020).

Methylotrophs are mainly strictly aerobic organisms, and according to the way that they use carbon substrate, they are divided into two taxonomic groups obligate and facultative methylotrophs. Obligate methylotrophic bacteria use one carbon compounds whereas, facultative methylotrophs are ubiquitous and use either one-carbon compounds or multi-carbon sources (De Marco et al., 2004; Mosin and Ignatov, 2014; Chistoserdova, 2018). Methane is a molecule that is extremely stable and challenging to chemically separate. However, a group of enzymes known as the methane monooxygenases (MMO), which found two types of it, soluble (sMMO) and particulate (pMMO). MMO is well recognized for having a wide substrate profile that includes several organic contaminants and may be used widely in the field of bioremediation (Khider et al., 2021).

The expression of each enzyme is significantly influenced by the extracellular copper content present in the growth medium. In contrast to sMMO, which includes iron (Fe), the pMMO enzyme fundamentally needs copper for its catalytic activity. (Semrau et al., 2018; Samanta & Sani, 2023). *Paenibacillus* members have unique physiological characteristics and have been shown to generate spores that are resistant to stress. They are surviving in various environments, and they have the ability to producing a numerous of bioactive substances. *Paenibacillus* species produce antibiotics, pesticides, or enzymes that are beneficial in bioremediation or the production of chemicals (Guan et al., 2023). The objective of current study was to characterize microbial communities that consume methane in contaminated soil samples and use it for the biodegradation of hydrocarbon compounds.

MATERIAL & METHODS

Soil Sample

Ten polluted soil samples were obtained from the surface (15 cm) near an oil well at Mushrif gas station in Basrah Governorate, Iraq in 2021. The soil samples were transferred to the laboratory in sterilized plastic bags and kept at room temperature until the microbiological screening.

Isolation of Methane-Oxidizing Bacteria

To isolate methanotrophic bacteria, 1 g of each soil sample was inoculated into 100 mL of nitrate mineral salt (NMS) medium, as described by Whittenbury et al. (1970). contained in a 100 mL glass bottle with a rubber stopper. After that 20 mL of methane was injected into each bottle using a 0.2µ filter syringe.

The bottles were incubated in an incubator shaker (Certomat, Sartorius- Germany) for 3- 4 weeks at 30°C and180 rpm, and examined every 3 days. To obtain single colonies, 0.1 mL of the growth medium was cultured on NMS agar for 4 weeks at 30 °C (Dianou and Adachi, 1999). Following incubation, the colonies were stained with Gram's stain and recognized under a microscope.

Identification of Bacterial Isolates

PCR technique has been followed up for identification of bacterial isolates. Geneaid Presto™ Mini gDNA Kit (Korea) was used consistent with the instructions of company to extract the genomic DNA from the bacterial isolates and the dideoxy Sanger method was used to sequence the 16S rDNA. Universal bacterial primers (Table 1) with a thermal cycler (Eppendorf, Germany) were used to amplify the 16S rDNA sequencing. 25 µL was used to conduct the reaction. The polymerase chain reaction (PCR) protocol consisted of an initial cycle of 95 °C for 5 min, after that 35 cycles of 30 s at 95 °C, 30 s at 55 °C, and 60 s at 72 °C, with a

Table 1. The sequence of synthesized oligonucleotide, company, origin and references

Gene	Primer	Sequence (5́-3́)	Company- origin	Reference
16S rDNA	F27	AGAGTTTGATCCTGGCTCAG	Macrogen - Korea	(Maki et al., 2024)
	R1492	GGTTACCTTGTTACGACTT		
pMMO	PmoC374degenF	AGCARGACGGYACNTGGC	Macrogen-Korea	(Ghashghavi et al., 2017)
	PmoA344degenR	ANGTCCAHCCCCAGAAGT		

final extension cycle at 72 °C for 5 min. The basic local alignment search tool (BLAST) was used to compare the sequences with nucleotide sequences deposited in the National Centre for Biotechnology Information (NCBI) database:

https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch.

Methane Monooxygenase Activity

This method was employed to assess the soluble monooxygenase activity in various bacteria based on their ability to convert naphthalene to 1-naphthol. The conversion was then observed colorimetrically by incorporating the aromatic diazo compound O-dianisidine into the reaction mixture. The bacteria were cultured in NMS medium without copper and 20% methane at 30 °C and 240 rpm for 7 days. To obtain colonies of different ages and sizes, the bacteria were incubated in NMS medium with 25% methane in an aerobic jar at 30 °C for 7 days (Graham et al., 1992). After incubation, 300–400 mg of naphthalene crystals was added to the plates' lids and next the plates were incubated for 60 min at 30 °C. To allow naphthalene to change into naphthol, 5 mg/L of O-dianisidine was added and given directly to the bacterial biomass.

Detection of pMMO Gene using qPCR

The Genezol™ TriRNA Pure Kit from Geneaid (Taiwan) was used to extract total RNA according to the manufacturer's instructions. A Nano Drop spectrophotometer was used to quantify the RNA in which triplicates were used to isolate the RNA from each sample. To generate cDNA, approximately 20 ng of RNA was reverse transcribed using the GoScript™ Reverse Transcription System (Promega, USA). Real time-PCR was performed using Go Taq® qPCR Master Mix from Promega (USA). A negative RT control sample was utilized to confirm that the extracted RNA samples were free of genomic DNA. The *pMMO* gene was amplified using the primer pairs (Table 1).

Growth with Crude Oil

The bacteria were grown in nutrient broth medium up to the logarithmic phase of growth, followed by centrifugation for 5 min at 8000 rpm. After discarding the supernatant, the pellets were hanging in sterile distilled water. This step was repeated three times. Subsequently, the bacteria were inoculated into flasks containing mineral salts medium (MSM) plus 1% crude oil, and incubated in a shaking incubator at 30 °C and 150 rpm for 10 days. Bacterial growth was monitored daily using a spectrophotometer (OD 600nm). Control flasks without bacteria were used (Su et al., 2023).

Analysis of Hydrocarbon Compounds Degradation

The residual crude oil was extracted using the liquid-liquid extraction method with a separating funnel. After discarding the aqueous phase, the residual oil was dried in an oven at

40 °C to eliminate carbon tetrachloride. The aliphatic and aromatic fractions were determined following the method described by Maki et al. (2023) and Maki et al. (2024). The aliphatic and aromatic compounds were collected and estimated using gas chromatography (GC, Agilent, UK). To assess the degradation rate, the GC profile of the hydrocarbon substrate was calculated as follows:

Biodegradation %= $(A-B)/A \times ^{(}$ "100" $)$ (Su et al., 2023).

A: represent the mass of petroleum hydrocarbon in the control, where B: is the mass of petroleum hydrocarbon left over after treatments been applied.

RESULTS AND DISCUSSION

Identification of Methane-Oxidizing Bacteria

In current study to isolate methane-oxidizing bacteria, oil-contaminated soil samples were collected from Mushrif gas station in Basrah Governorate southern Iraq. Where methane is emitted as a result of the incineration of oil and gas at a rate of 25% (Collins et al., 2022). Facultative methanotrophic bacteria grow on methane or other compounds (methyl alcohol, methylamine, dimethyl amine, formate, and formaldehyde) as their only source of carbon, while obligate methanotrophic bacteria grow only on methane, many bacteria have been discovered that consume methane from various environments (Rani et al., 2021; Macey et al., 2020).

The isolated bacteria were molecularly identified by 16S rDNA sequencing and showed over 99% similarity to *Paenibacillus lautus* and *Paenibacillus* sp in the NCBI database. Apparently, *P. lautus* has the ability to consume methane gas and other hydrocarbon compounds as the sole source of carbon and energy. To the best of our knowledge there are no studies globally on the ability of *P. lautus* to oxidize methane. In a study by Mauricio-Gutiérrez et al. (2020), they isolated *Paenibacillus* sp and *Bacillus* sp from agricultural soil contaminated with diesel.

Methane Monooxygenase Activity

No positive results were obtained for sMMO activity in free copper medium, indicating the existence of PMMO enzyme. While Rani et al. (2021) discovered *Paenibacillus* sp showed positive results for sMMO activity.

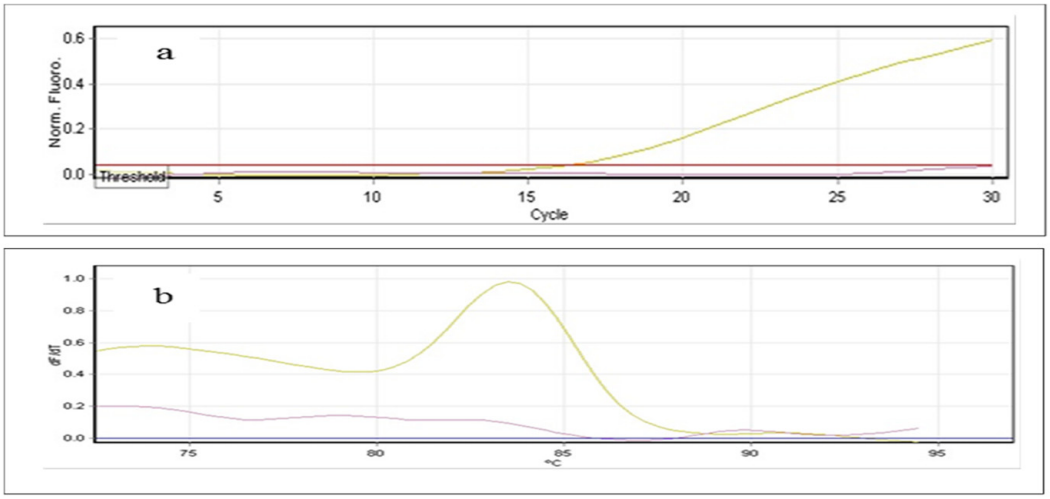

Fig. 1. a) Amplification curve analysis of *pMMO* gene expression for 1 replicating of *Paenibacillu*s sp., the signal was detected at the CTs range. b/ Melting curve analysis of the *pMMO* gene expression revealed the degree of plot specificity in one peak at (84 °C).

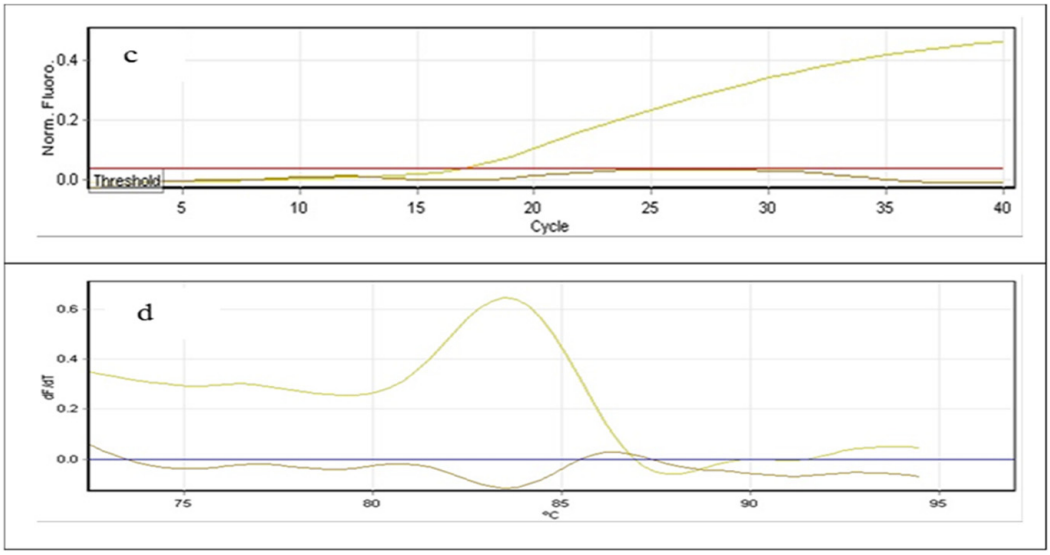

Fig. 2. c) Amplification curve analysis of *pMMO* gene expression for 1 replicating of *Paenibacillus lautus* the signal was detected at the CTs range. b/ Melting curve analysis of the *pMMO* gene expression revealed the degree of plot specificity in one peak at (84 °C).

Detection of pMMO Gene Using qPCR

pMMO gene was detected using isolated RNA as a template for the reverse transcription of the separated RNA into cDNA. This process allowed for the gene's detection. To initiate the reaction, the cDNA, a primer specific for pMMO and SYBR green dye were mixed together (Fig.1, 2). This is responsible for encoding the methane monooxygenase enzyme and might explain the ability of methylotrophs to use methane in NMS medium. This result agrees with Jhala et al. (2014) who finding that *Paenibacillus illinoisensis* isolates may possess the pMMO enzyme.

Growth with Crude Oil

The bacteria that have highest light intensity were selected for the oil bio cracking experiment. All bacteria were grown on MSM containing1% crude oil. The optical density of *Paenibacillus* sp was 1.9 after 5 days of incubation, while the OD of *P. lautus* was 0.5 after 5 days of incubation (Fig 3, 4). The degradation of hydrocarbon compounds is evaluated by increasing the number of bacterial cells, which indicates their capability to utilize only these compounds. The study of growth features showed that *P. lautus* and *Paenibacillus* sp could grow in MSM containing 1% (v/v) crude oil. These results are consistent with Maki et al. (2023), who used crude oil for growth *Methylorubrum extorquens*. The bioremediation to be effective, the contaminants must be broken by microorganism enzymes and convert it into harmless molecules. Its application often requires overcoming natural barriers to allow more rapid growth of microbial development, as it is effective when ecological conditions promote microbial development and migration (Trimurtulu, 2021).

Analysis of Petroleum Hydrocarbon Degradation

Degradation Rate of n-alkanes in Crude Oil

Proportion of degradation of n-alkane (C_{10}–C_{40}) available in crude oil by *Paenibacillus* sp and *P. lautus* is displayed in Figure 5 (b and c). Both bacteria capably degraded all n-alkanes,

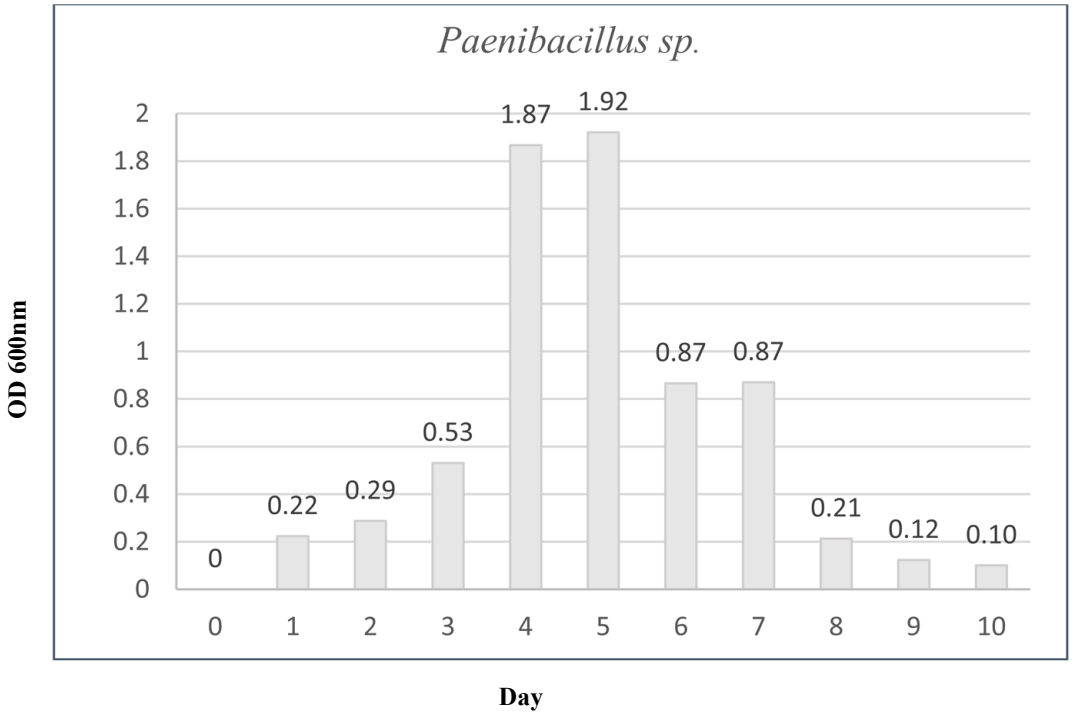

Fig. 3. Optical density of *Paenibacillus* sp. over 10 days of incubation at 1% crude oil.

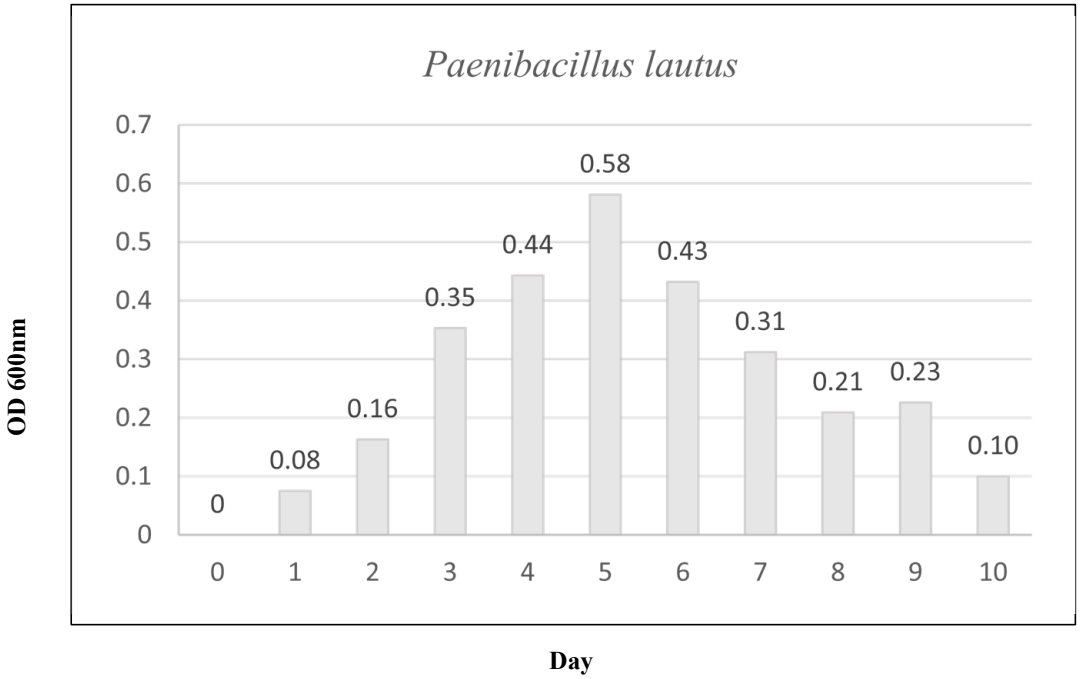

Fig. 4. Optical density of *Paenibacillus lautus* over 10 days of incubation at 1% crude oil.

approximately 92.8% and 89.41%, respectively, and were compared with the control, which contains only crude oil without bacteria (Fig. 5, a). This degradation rate is higher than the 45.9% in aliphatic fractions after 7 days reported for *Paenibacillus ehimensis* isolated from oil contaminated soil samples in Oman (Shibulal et al., 2017). Furthermore, Mauricio-Gutiérrez

et al. (2020) found that *Bacillus pumilus* and *P. lautus* degraded and used diesel as the only carbon source. The concentrations of degraded diesel by *Bacillus pumilus* was 24,000 mg /L, meanwhile the degradation of *Paenibacillus lautus* was lower.

Degradation Rate of PAH in Crude Oil

Paenibacillus sp and *P. lautus* were efficiently degraded all PAH after 5 days of incubation with 1% ($^{V}/_{V}$) crude oil, approximately 98.8% and 97.28%, respectively (Fig. 6, b and c) and

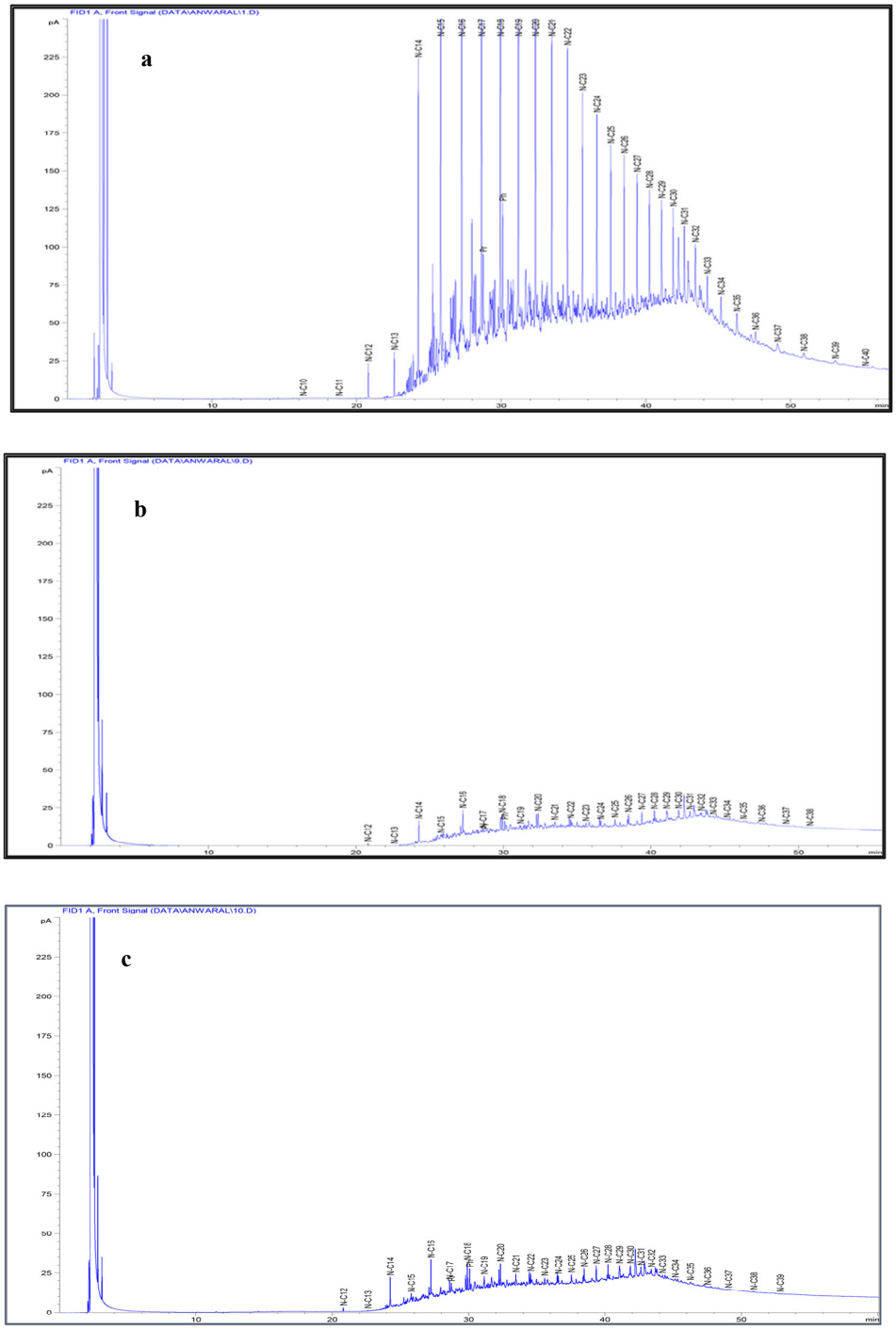

Fig. 5. GC chromatograms of aliphatic compounds appear the control (a) and the changes of biodegradation using *Paenibacillus* sp; (b) and *Paenibacillus lautus* (c).

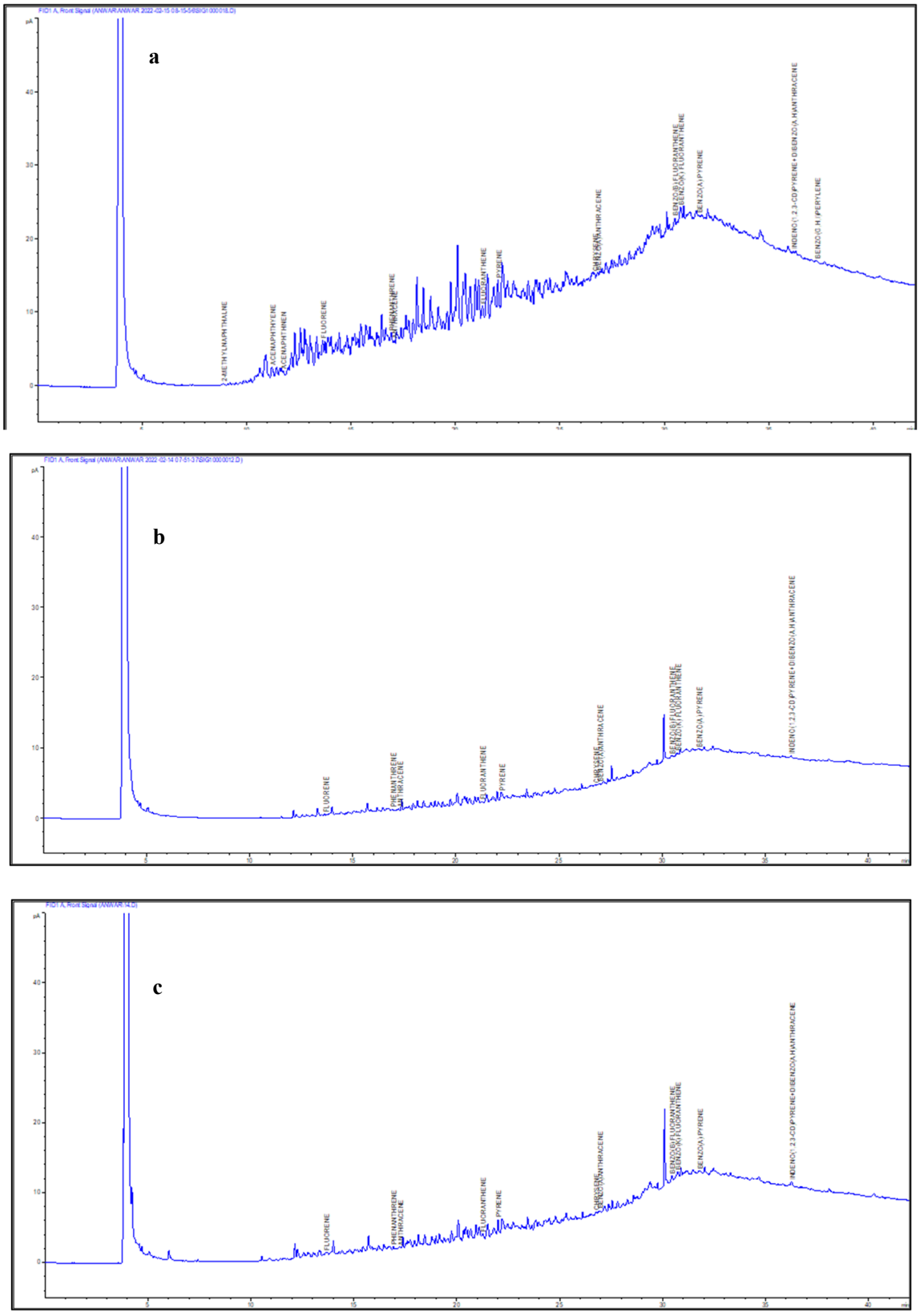

Fig. 6. GC chromatograms of aromatic compounds appear the control (a) and the changes of biodegradation using *Paenibacillus* sp; (b) and *Paenibacillus lautus* (c).

were compared with the control, which contains only crude oil without bacteria (Fig 6a). These results are agreed with Shibulal et al., (2017) who found that *Paenibacillus ehimensis* has the ability to degrade PAHs into aromatic fractions of 85.3%. *Paenibacillus* produced numerous enzymes that metabolize aliphatic and aromatic organic pollutants, including oxygenases, dehydrogenases, and ligninolytic enzymes (Grady et al., 2016). Methylotrophic bacteria have the potential to degrade a significant amount of aromatic contaminants because of the presence of MMO enzyme (Giri et al., 2021). This high degradation may be due to the fact that bacteria was isolated from oil spill sites, in accordance to Hamad et al. (2021) who recommended that bacteria isolated from oil-contaminated sites could degrade petroleum hydrocarbons more effectively than sites without oil contamination.

CONCLUSIONS

Paenibacillus bacteria have the ability to grow on NMS medium containing methane as the sole source of carbon and energy; furthermore they have the methane monooxygenase enzyme. So, *Paenibacillus* bacteria are capable of bioremediating crude oil and can be used to clean polluted environments.

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The present research did not receive any financial support.

CONFLICT OF INTEREST

The authors declare that there is not any conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/ or falsification, double publication and/or submission, and redundancy has been completely observed by the authors.

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