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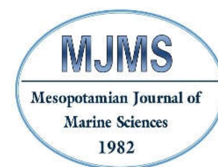

Measuring the Degradation of Aromatic Compounds Using Methylorubrum extorquens Isolated from Oil-Contaminated Soils in Southern Iraq

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Polycyclic aromatic hydrocarbons,
qPCR

Abstract - An innovative, inexpensive, and environmentally acceptable approach is to use microbes to degrade hazardous materials to reduce soil pollution. Therefore, the purpose of this study was to identify and assess the performance of bioremediation bacteria in soil located adjacent to oil fields. Soil samples were collected, diluted, and grown on a mineral selective medium supplemented with methanol as the sole carbon source. Based on the phenotypic, biochemical, and molecular characteristics, seven out of fifteen isolates were identified as *Methylorubrum extorquens*. This isolate displayed a high growth rate on methanol, with optical density values of 1.9 at 1% concentration and 1.8 at 2% concentration. Furthermore, it can grow in crude oil as an energy and carbon source. It exhibited a density of 0.09 after seven days of incubation. To our knowledge, this study is the first to report the ability of *Methylorubrum extorquens* to grow on crude oil and degrade polycyclic aromatic hydrocarbons at a ratio of 65.69% of (v/v) crude oil 0.5%. Due to its presence in contaminated soils, the isolated strain has potential as a natural biodegradation tool to eliminate contamination in oil-related compounds.

قياس تحلل المركبات العطرية بواسطة *Methylorubrum extorquens* المعزولة من التربة الملوثة بالنفط في جنوب العراق

أنوار عبد الوهاب مكي[1] ، أسعد محمد رضا الطائي[1] ، زينة وحيد عطوان[2]
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المستخلص – ان استخدام الميكروبات لتكسير المواد الخطرة لتقليل تلوث البيئة هو نهج مبتكر وغير مكلف ومقبول بيئيا. لذلك هدفت الدراسة إلى عزل وتقييم كفاءة البكتيريا للمعالجة الحيوية في التربة القريبة من حقول النفط. تم جمع عينات التربة وتخفيفها وتنميتها على وسط انتقائي معدني مكمل بالميثانول كمصدر وحيد للكربون. بناءً على الخصائص المظهرية والكيميائية الحيوية والجزيئية، تم عزل 15 عزلة منها سبع سلالات تم تحديدها على أنها *Methylorubrum extorquens* أظهرت هذه العزلة معدل نمو مرتفع على الميثانول، بقيم كثافة بصرية 1.9 بتركيز 1% و 1.8 بتركيز 2%. علاوة على ذلك، فقد نمت بالاعتماد فقط على النفط الخام كمصدر للطاقة ومصدر للكربون. أعطت كثافة 0.09 بعد 7 أيام من الحضانة. على حد علمنا، عالمياً هذا هو التقرير الأول عن قدرة*Methylorubrum extorquens* على النمو على النفط الخام وتحلل الهيدروكربونات العطرية متعددة الحلقات بنسبة 65.69 % من النفط الخام 0.5% حجم / حجم. نظراً لوجودها في التربة الملوثة، يمكن أن تكون السلالة المعزولة أداة طبيعية محتملة للتحلل البيولوجي للقضاء على التلوث بالمركبات المرتبطة بالزيت.

الكلمات المفتاحية: المعالجة الحيوية، نفط خام، ميثانول، تفاعل البوليميراز المتسلسل الكمي.

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Introduction

Oil refinery wastes and unintended oil spills are two major environmental issues caused by the widespread use of crude oil and petroleum. In particular, polycyclic aromatic hydrocarbons (PAHs) are relatively resistant to biodegradation, while some crude oil components degrade easily. Some PAHs are categorized as high-priority pollutants, which are dangerous to both people and wildlife because of their mutagenic and carcinogenic potentiality (Medić *et al.*, 2020). Given that hydrocarbons could be integrated into food chains, reducing their harmful effect on the environment is currently a key objective in global research (Taher & Saeed, 2021).

Chemical, physical, and thermal treatments are among the methods that have been proposed for eradicating petroleum contamination. However, these approaches are often impractical and even environmentally harmful because of the resulting by-products (El-Sheshtawy *et al.*, 2022). Biological techniques allow a total breakdown of oil into CO_2 and H_2O, and they are more practical, affordable, and simple to use. They are also eco-friendly since they do not destroy microorganisms in the soil or harm the soil structure (Fadhil & Al Baldawi, 2020; Lopez-Echartea *et al.*, 2020).

Additionally, bioremediation requires prolonged treatment and might not be effective if there are considerable concentrations of pollutants that are harmful to microorganisms.

Enzymes, which are created by microorganisms in the presence of carbon sources, are responsible for the biodegradation of petroleum hydrocarbons. On the other hand, the absence of suitable enzymes acts as a barrier in the hydrocarbon degradation process (Overton *et al.*, 2019).

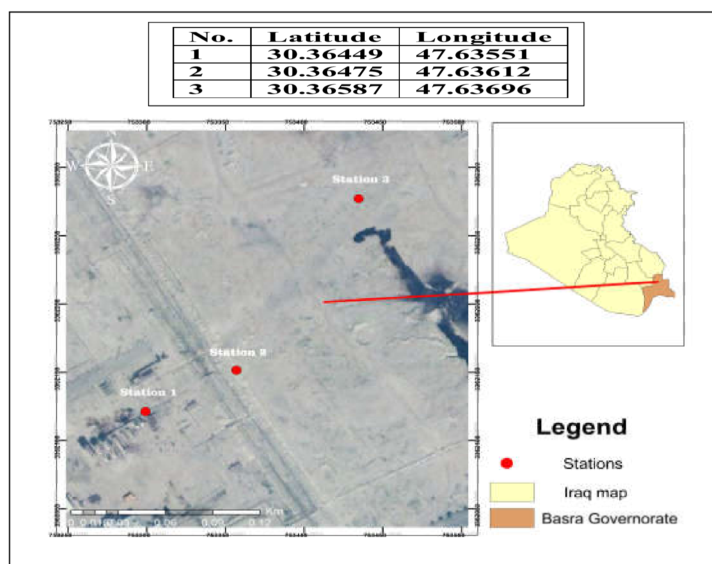
Methylotrophs have the ability to utilize single-carbon compounds as a source of carbon for growth and are crucial to the cycling of single-carbon compounds (Yang *et al.*, 2020). Methylotrophic bacteria may survive in a wide range of environmental conditions, such as low temperatures, hyper-saline conditions, and droughts, as well as acidic and alkaline habitats (Kumar *et al.*, 2019). According to reports, *Methylobacterium* sp. can biodegrade various pollutants, including explosives, Methyl tert-butyl ether (MTBE), and PAH (Amaresan *et al.*, 2020).

Since these microbes break down and mineralize organic xenobiotic substances, they could potentially be used in bioremediation procedures. They also have potential synthetic biology applications, which enables their genetic modification (Camargo-Neves and Araújo, 2019). This study aims to determine the efficiency with which *Methylorubrum extorquens* degrades polycyclic aromatic hydrocarbons.

Material and Methods

Sample Collection

Three samples (25g) were collected from the Al-Zubair oilfield in Basrah, southern Iraq during December 2020. The samples were immediately processed in the laboratory and submitted to identify the growing bacteria and evaluate their biodegradation capacity (Figure 1).



No.	Latitude	Longitude
1	30.36449	47.63551
2	30.36475	47.63612
3	30.36587	47.63696

Figure 1. Al-Zubair oilfield sampling stations 1, 2, and 3

Isolation and Identification of Methylotrophic Bacteria

A methanol salt medium (MSM) for growth consists of KNO_3, 2 g; $MgSO_4.7H2O$, 0.2 g; $FeSO_4$., 10mg; Na_2HPO_4, 0.21 g; KH_2PO_4, 0.09 g; $CuSO_4.5H_2O$, 0.5 g; H_3BO_3, 30 mg; $MnSO_4.5H_2O$, 2 mg; $ZnSO_4.7H_2O$, 10 mg; $Na_2MoO_4.2H_2O$, 3 mg and fluconazole 0.05 mg (Kouno *et al.*, 1973). The medium was dissolved in 1 litre of distilled water and the pH was adjusted to 6.8. The fluconazole and 10 ml of methanol were added after sterilization. To improve the growth rate, a shaking incubator (180 rpm) was used to incubate the soil samples (0.5 g) in basal methanol medium for seven days at 30 °C.

Morphological and Biochemical Analysis

In addition to Gram staining, which was used to disclose any microscopic traits, the most essential morphological criteria assessed were a pink tint and the form of the colony. Finally, tests involving oxidase and catalase were conducted as biochemical examinations.

DNA Extraction and Identification of Bacteria using 16S rRNA

Following the manufacturer's instructions, DNA was extracted from the bacterial isolates using the Presto^{TM} Mini gDNA bacteria kit (Geneaid, Taiwan, Cat. No. GBB100). The concentrations and quality of all the genomic DNA samples were measured using the DNA-RNA Spectrophotometer (Optizen, Korea). A gene-specific primer pair was used to amplify 16S rRNA genes, F27(5́-AGAGTTTGATCCTGGCTCAG-3́) and R1492 (5́-GGTTACCTTGTTACGACTT-3́) (Abd Al Wahid and Abd Al-Abbas, 2019).

Each PCR reaction was conducted in a 50µl volume containing 2 µl (10-20 ng) of DNA template, 2 µl of each primer, 25 µl of Go Taq Green master mix (Promega, USA), and 19 µl of nuclease-free water. The following conditions were used for the PCR reaction: 95 °C for 5 min, followed by 35 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 60 s, with a final extension at 72 °C for 5 min. All the expected PCR products were sent to the Macrogen Company (South

Korea) for sequencing following visualisation on a 2% agarose gel. The Basic Local Alignment Search Tool (BLAST) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) was used to align the query sequences of each sample against the database nucleotide subject sequences.

RNA Extraction

Both *M. extorquens* and *Pseudomonas balearica* grew exponentially in a minimal medium using methanol as the sole carbon source. In accordance with the instructions, the GENEzol™ TriRNA Pure Kit (Geneaid, Taiwan, Cat. No. GZXD100) was used to extract the total RNA. Three biological replicates were used to isolate the RNA from each isolate, and the quantity and quality of the RNA were measured using the NanoDrop spectrophotometer.

RT-qPCR Evaluation

The GoScript™ Reverse Transcription System (Promega, USA) and a random primer were exploited to synthesize the cDNA. qPCR was conducted using the real-time PCR amplification system (Bioneer Korea + rotor gene) and Go Taq® qPCR Master Mix (Promega, USA). 16S rRNA encoding was used as a reference gene. Following the manufacturer's directions, RT-qPCR was carried out. Three replicates of each reaction were run.

The relative quantification of the RT-qPCR experiments was analyzed using the $2^{-\Delta\Delta Ct}$ technique. The resulting Ct values served as the initial data used to calculate the relative expression level of the target genes, which was then normalized against that of the 16S rRNA gene. A non-template sample was used to guarantee the absence of any cross-contamination. The MxaF and 16S rRNA genes were amplified using 1004F (5́-CCWGGYGAYAAYAARTGGTC-3́) and 1232R (5́-GCCCAGTTNAYRAAKGGRTG-3́) (Ramachandran and Walsh, 2015) and 16S-Fw (5́-TGAGATGTTGGGTTAAGTCCCGCA-3́) and 16S-Rv (5́-CGGTTTCGCTGCCCTTTGTATTGT-3́), respectively (Zhang *et al.*, 2016).

The thermal program for MxaF was set as follows: 96 °C for 5 minutes, then 40 cycles at 96 °C for 30 s and 56 °C for 60 s, followed by an extension at 72 °C for 30 s. Meanwhile, the real-time PCR program for the 16S rRNA primer was optimized as follows: an initial denaturation was achieved at 94 °C for 3 min, followed by the second step of 45 cycles for each (denaturation at 94 °C for 30 s, annealing at 52 °C for 30 s, and the extension at 72 °C for 40 s). To ensure the specificity of the selected primers, the reaction was subjected to melting curve analysis.

Growth at Different Methanol Concentrations (1–10) %

A single colony was grown on the MSM broth; the growth was examined continuously until the logarithmic phase had been reached by measuring the optical density (OD) at 600 nm using a spectrophotometer. To determine the ability of bacteria to grow at different methanol concentrations, they were grown in concentrations of 1-10% methanol for a 10-day period, and their daily growth was monitored using a spectrophotometer.

Growth in Crude Oil

To obtain a pure colony of bacteria, a 1:100 (v/v) ratio of MSM broth to crude oil was utilised, with crude oil serving as the only source of carbon. A daily comparison was made between the bacterial growth on the MSM and that on a negative control consisting of MSM and crude oil, based on the measurement of OD_{600}. This comparison was conducted over seven days.

Biodegradation of PAHs by Methylotrophic Bacteria

Bacteria were taken in log phase and grown in 100 ml of MSM broth and 0.5 ml of crude oil using a shaker incubator (120 rpm) at 30 °C. Each experiment was repeated twice, with both times yielding exactly the same results (Al-Taee *et al.*, 2017).

Analysis of PAHS by gas chromatography

A liquid-liquid extraction method was used to extract the residual crude oil using a 500 ml separating funnel, and it was oven-dried at 40 °C after eliminating the aqueous phase containing CCl_4. The aromatic fraction was extracted and the remaining crude oil was dissolved in 25 ml of benzene (Alkanany *et al.*, 2017). The extraction process for the control flasks was similar, while the FID gas chromatography technique was used to estimate the percentage of aromatic hydrocarbons (Agilent Chem Station).

Results

Isolation and Identification of Methylotrophic bacteria

Using a newly modified medium, seven strains were isolated from polluted soil. The bacteria were identified as gram-negative, rod-shaped, in mass, non-spore-forming, catalase, or oxidase-positive. A key identifying characteristic was their ability to produce colour pigments and molecular detection.

Identification of Bacteria using 16S rRNA

The PCR product was electrophoresed into 2% agarose, and the 16S rRNA target area was amplified to a size of 1,500 base pairs so it could be used as a target. The BLAST analysis concluded with a 99.9% degree of identification that the isolated bacteria belonged to the species *Methylorubrum extorquens* and *Pseudomonas balearica*. *P. balearica*, served as control organisms, and had been collected from the same setting as the other samples.

Comparison of Gene Expression of *MDH* in *M. extorquens*

To compare the expression of the *MDH* gene in *M. extorquens* compared to the other species isolated in the same study (the control), real-time PCR using SYBR green dye and *MDH* gene-specific primers were employed. The *MDH* expression upregulated 44-fold compared to the control sample, which was normalized to 1. ΔΔ. CT analysis was applied to conclude the *MDH* gene expression. The 16S rRNA value was subtracted as the housekeeping gene (Figures 2 and 3).

Growth at Different Methanol Concentrations (1–10) %

Methanol is the only carbon and energy source used by methylotrophic bacteria like *M. extorquens*. It interacts with biological components as an alcohol solvent, modifying their structure and chemical stability in a concentration-dependent manner. *M. extorquens* showed maximum growth at 1% and 2% methanol, while the growth reduced in concentrations of 3-6% and no growth was found in concentrations of 7-10% (Figure 4).

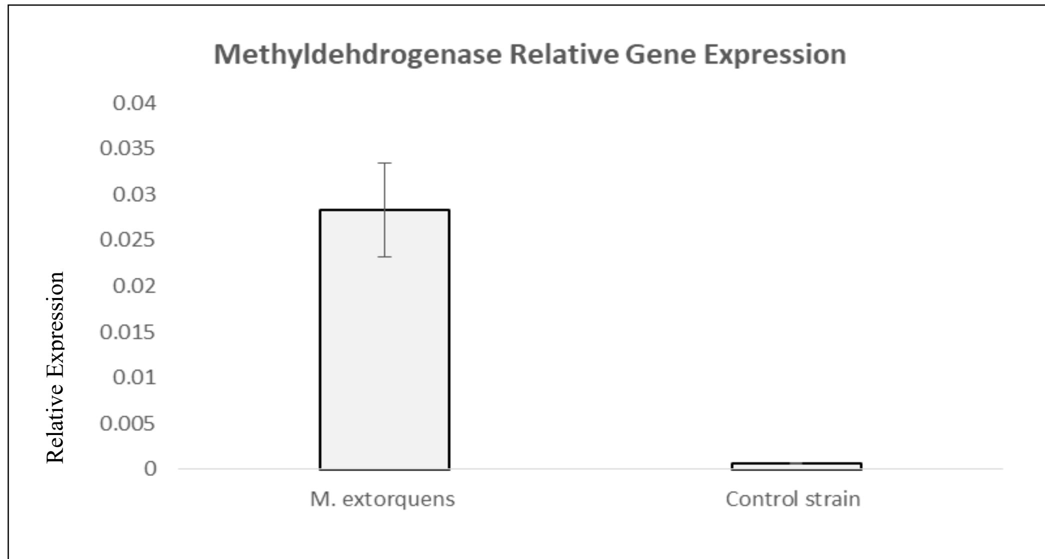


Figure 2. Relative Methyl Dehydrogenase gene expression in *M. extorquens* and control strain (*Pseudomonas balearica*) bacteria. Total RNA was extracted from fresh cultures of both species, reverse transcription of the synthesized DNA was performed, and the synthesized DNA was employed as a template in a quantitative polymerase chain reaction (qPCR) relative expression experiment using SYBR green master mix. Analyses were performed using ΔΔ CTs, and results were normalized using 16S rRNA as the housekeeping gene.

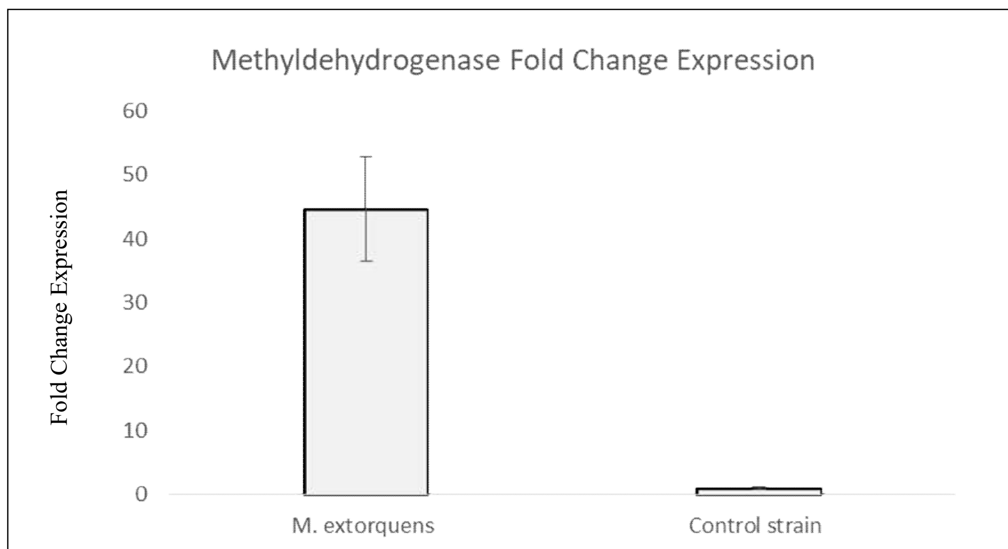


Figure 3. Methyl Dehydrogenase fold change analysis in *M. extorquens* and control strain (*Pseudomonas balearica*). Total RNA was extracted from fresh cultures of both species, reverse transcription of the synthesized DNA was performed, and the synthesized DNA was employed as a template in a quantitative polymerase chain reaction (qPCR) relative expression experiment using SYBR green master mix. Analyses were performed using ΔΔ CTs, and results were normalized using 16S rRNA as houses keeping gene and control sample was normalized to 1.

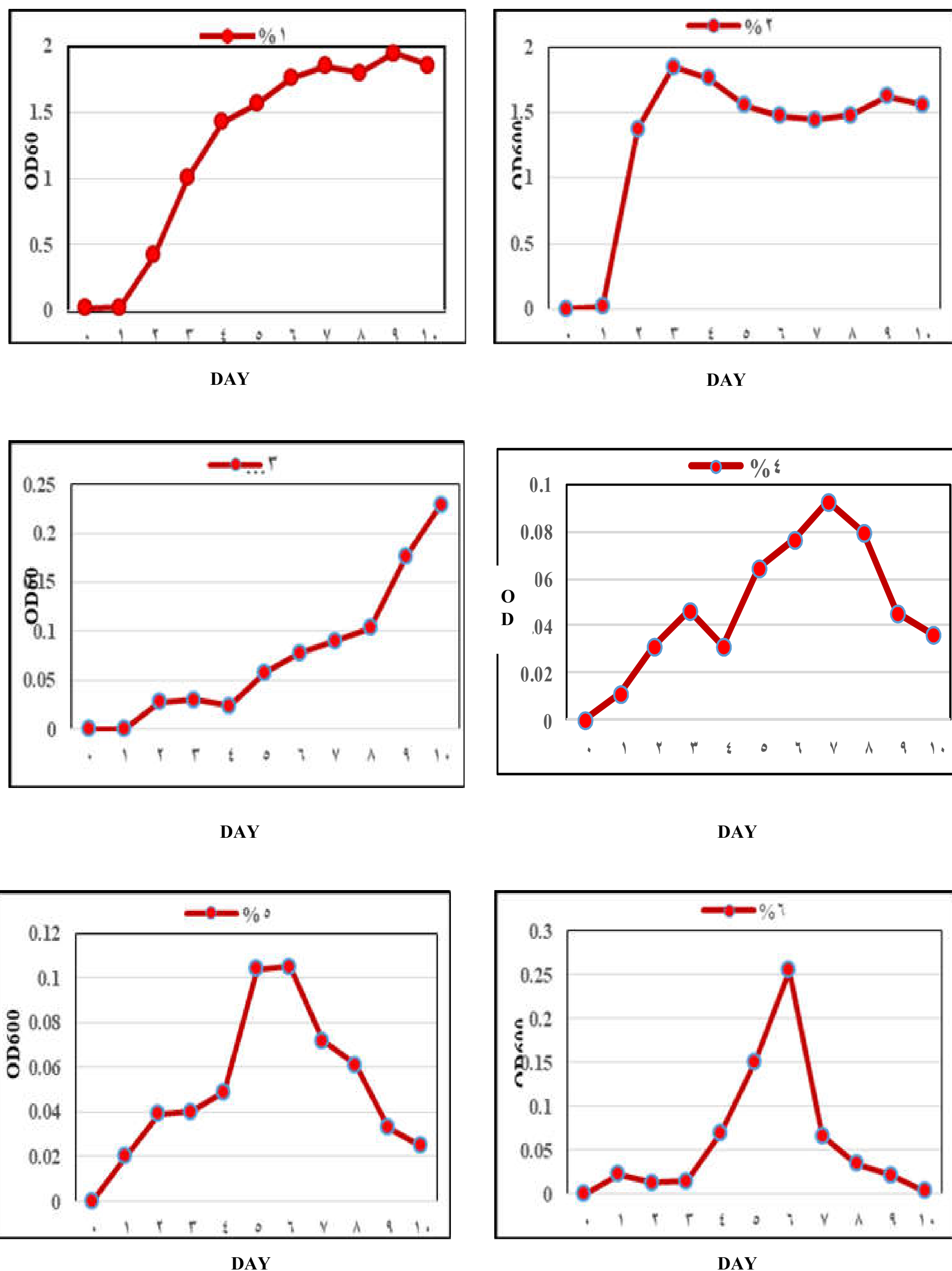


Figure 4. Growth of *M. extorquens* on salt medium supplemented with increasing concentrations of methanol. The medium was supplemented with 1%, 2%, 3%, 4%, 5% and 6%, methanol.

Growth in Crude Oil

The current study shows that *M. extorquens* can grow in crude oil, which contains a wide range of different hydrocarbon types derived from petroleum products. After seven days of incubation in the MSM media with 1% crude oil, the turbidity was clearer and the OD_{600} was 0.097 (Figure 5).

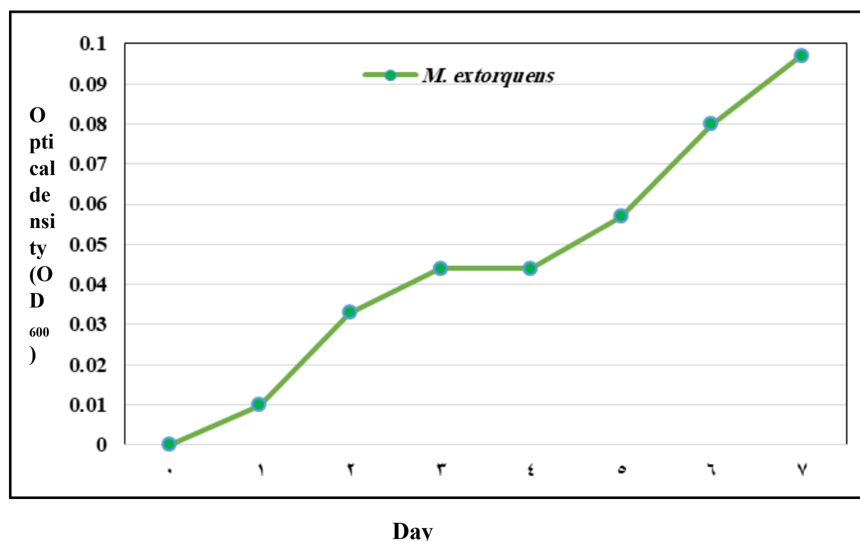


Figure 5. The optical density of *M. extorquens* growth with 1% crude oil after 7 days of incubation.

Biodegradation of PAHs by *M. extorquens*

Analyses using the GC device proved that *M. extorquens* growing in a medium containing 0.5% v/v could break down aromatic compounds at a ratio of 65.69%.

where the degradation percentages for phenanthrene, Benzo(A) Anthrac, Chresene, Fluorene, Benzo(B) Fluora, and Benzo(A) Pyrene were 81.81% and 75.35%, 71.71%, 70.13%, 69.09%, and 60.01%, respectively. Meanwhile, the 2-methylnaphtha and acenaphthene fractions were completely degraded (Figures 6 and 7).

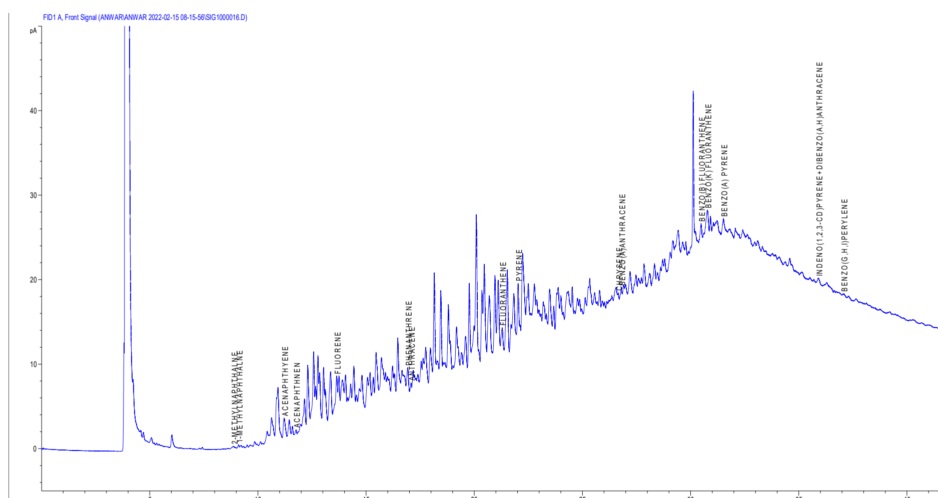


Figure 6. GC of residual control crude oil (aromatic portion) 0.5 % (v/v) after 7 days of incubation.

Figure 7. GC of residual crude oil (aromatic portion) of 0.5 % (v/v) after 7 days of incubation of *M. extorquens*.

Discussion

The common bacteria that grow in modified MSM are methylotrophic, depending on their ability to exploit methanol as the sole source of energy (Belkhelfa *et al.*, 2019). Seven of the fifteen gram-negative bacilli were identified by 16S rRNA as methylotrophic bacteria. These findings are supported by the conclusion of Rojas-Gätjens *et al.* (2022) , who discovered several methylotrophic bacteria in an abandoned oil well, including *Paracoccus communis* and *Methylorubrum rhodesianum*. *M. extorquens* showed the ability to grow in methanol or crude oil as a source of carbon and energy. In addition, they had methanol genes encoding the methanol dehydrogenase enzyme. According to Green & Ardley (2018), this was reclassified and given the name *Methylorubrum*. This was achieved using phenotypic data, genomic data, the16S rRNA gene sequence, and multi-locus sequence analysis (MLSA).

Since *M. extorquens* has an XoxF encoding region and *P. aeruginosa* showed the ability to express methyldehydrogenase, the latter has the ability to oxidise N- methyl l-glutamate, which was attributed to the presence of respiratory chain-linked dehydrogenase. Mutant insertion analysis revealed that *P. aeruginosa* could catalyse methyl-branched compounds (Förster-Fromme & Jendrossek, 2008; Hersh *et al.*, 1971). Gene expression analysis showed that the enzyme activity in *M. extorquens* was a 44-fold change compared to its expression in *P. balearica*. This could be attributed to the fact that the enzyme was clearly represented in the *M. extorquens* genome and could be expressed at its highest level when the substrate was efficiently provided; meanwhile, in *P. balearica*, it is part of its cytochrome chain reactions.

The results illustrated the ability of bacteria to grow in varying methanol concentrations (1-6) %. In line with the findings of Kim *et al.* (2003), as the methanol concentration was raised, the specific growth rate fell, while at a 6% methanol concentration, growth was significantly suppressed. A 0.5% methanol concentration was determined to be optimal for producing the maximum specific growth rate. This may be due to the presence of the methanol dehydrogenase enzyme, which converts methanol to formaldehyde, which is toxic to cells. Nevertheless,

methylotrophs have a built-in resistance to the toxic effect of formaldehyde (Khider *et al.*, 2021). *M. extorquens* proved its ability to grow on crude oil as a source of carbon and energy. This result is in agreement with a study by Godini *et al.* (2018), who found that *Methylobacterium persicinum* could thrive in mineral salt broth, with 2% crude oil as the only source of carbon and energy.

The long-term growth of *M. extorquens* enables it to break down 65.69 % of crude oil. This may be due to the harsh environment from which the bacteria were isolated. To our knowledge, this is the first time that *M. extorquens* has been introduced as a species with the ability to degrade PAH compounds. This study is supported byVentorino *et al.* (2014) who found that a *Methylobacterium populi* VP2 strain could degrade polycyclic aromatic hydrocarbons (PAH) in a contaminated environment. Another study by Salam *et al.* (2015) examining the bacterium *Methylobacterium mesophilicum* revealed that it has the capacity to break down PAH components, including Anthracene and Pyrene.

Conclusions

This study presents the first investigation of *Methylorubrum extorquens* growing in crude oil as the sole carbon and energy source, as well as degrading PAH compounds. The 16S rRNA gene sequencing data, the existence of the *MxaF* gene encoding methanol dehydrogenase, and the isolation of bacteria able to use single-carbon molecules provide evidence of the presence of methylotrophic bacteria in oil soil. Despite the current knowledge about bacteria that break down hydrocarbons, it is recommended that rather than using a single type, a variety of bacterial species should be used to speed up biodegradation.

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