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## Molecular Identification of *Methylobacterium extorquens* using PCR-Amplified *MxaF* Gene Fragments as A Molecular Marker

Anwar A. Maki<sup>1</sup> 

Asaad M. R. Al-Tae<sup>1\*</sup> 

Zeenah Weheed Atwan<sup>2</sup> 

<sup>1</sup>Department of biological Development, Marine Science Center, Basra University, Basra, Iraq.

<sup>2</sup>Department of Microbiology, College of Medicine, University of Basra, Basra, Iraq.

\*Corresponding author: [amrtaee@yahoo.com](mailto:amrtaee@yahoo.com)

E-mail addresses: [anwar.maki@uobasrah.edu.iq](mailto:anwar.maki@uobasrah.edu.iq) , [zeenah.atwan@uobasrah.edu.iq](mailto:zeenah.atwan@uobasrah.edu.iq)

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### Abstract:

Methylotrophs bacteria are ubiquitous, and they have the ability to consume single carbon (C1) which makes them biological conversion machines. It is the first study to find facultative methylotrophic bacteria in contaminated soils in Iraq. Conventional PCR was employed to amplify *MxaF* that encodes methanol dehydrogenase enzyme. DNA templates were extracted from bacteria isolated from five contaminated sites in Basra. The gene specific PCR detected *Methylobacterium extorquens* as the most dominant species in these environments. The ability of *M. extorquens* to degrade aliphatic hydrocarbons compound was tested at the laboratory. Within 7 days, gas chromatographic (GC) studies of remaining utilized crude oil revealed that 61.14 % of the initial content had been degraded, and GC fingerprinting of the utilized aliphatic compounds revealed significant reductions in C<sub>12</sub>, C<sub>13</sub>, C<sub>14</sub>, and C<sub>15</sub>. Globally this is the first time found a new strain of *M. extorquens* has the ability to degrade aliphatic hydrocarbons compound. Conventional PCR and gene sequencing revealed the presence of the facilitative methylotrophic bacteria in polluted areas in Basra. *M. extorquens* was dominant and showed a substantial ability to degrade crude oil which makes them an important tool to be employed in bioremediation.

**Keywords:** gas chromatography, Methanol dehydrogenase, *Methylobacterium extorquens*, Methylotrophs bacteria, *MxaF* gene.

### Introduction:

Various species of methylotrophic bacteria are distributed in nearly all natural environments<sup>1</sup>. Methylotrophs use reduced single-carbon (C1) molecules like methanol as carbon sources for growth, making them methanol biological convertors<sup>2</sup>. More than 50 methylotrophic taxa have been identified, including Alpha, Beta, and Gamma-proteobacteria, Verrucomicrobia, Firmibacteria, Actinobacteria, and Flavobacteria<sup>3</sup>.

Pink-pigmented facultative methylotrophs (PPFMs), are present in the rhizosphere or exist in soil, air, or water. PPFMs belong to methylbacteriaceae family, they are gram negative and utilize C1 molecules as their only source of energy and carbon, methanol, methylamine, formate, and formaldehyde are among the examples<sup>4</sup>.

*Methylobacterium* has more species than any other within the family methylbacteriaceae, order rhizobiales, and class alphaproteobacteria. *Methylobacterium* includes facultative methylotrophs that may live on carbon and energy

sources other than organic acids and sugars, such as methane or methanol<sup>5</sup>. All other previously PPFMs bacteria were classified within *Methylobacterium* in a taxonomic analysis<sup>6</sup>. Following that, according to 16S rRNA gene sequences, multi-locus sequence analysis (MLSA), phenotypic data, and genomics, eleven *Methylobacterium* species were reclassified as *Methylobacterium*, a new genus<sup>7</sup>.

Methanol dehydrogenase (MDH) converts the methanol to formaldehyde, which is the second crucial enzyme in methane metabolism. The MDH is a pyrroloquinoline quinone (PQQ)-containing soluble periplasmic enzyme with a  $\alpha_2\beta_2$  structure, consisting of two large subunits *MxaF* and two tiny subunits *MxaI*, the active site contains a Ca<sup>2+</sup> ion<sup>8</sup>.

*MxaF* encodes for the large alpha-subunit of MDH and other functional molecular marker genes are highly conserved across methylotrophs and have been utilized in environmental studies to identify methylotrophs in different environments<sup>9</sup>.

One of the most significant environmental pollutants, with bad impacts on both people and the environment is hydrocarbons<sup>10</sup>. It is a crucial global environmental pollutant because it spills and leaks often throughout the exploration, transport, refining, and storage of petroleum and petroleum products. The fundamental approach for reducing biodegradable contaminants is biodegradation, which is cost effective alternative<sup>11</sup>. It is one of the most effective and promising methods for cleaning up soil that has been contaminated with diesel. This choice has the potential to remove harmful contaminants through biological activity<sup>12</sup>.

The aim of this study is to isolate methylotrophic bacteria from hydrocarbon-contaminated soils. Identification of these bacteria according to their morphological features and 16S rRNA gene sequencing. Determination of their capability to degrade aliphatic compounds *in vitro*.

## Material and Methods:

### Sampling

A total of 10 g of oil-polluted soil was collected aseptically in sterile plastic bags from five oil sites in Basra city, southern Iraq in December 2020, Fig. 1 and Table 1.

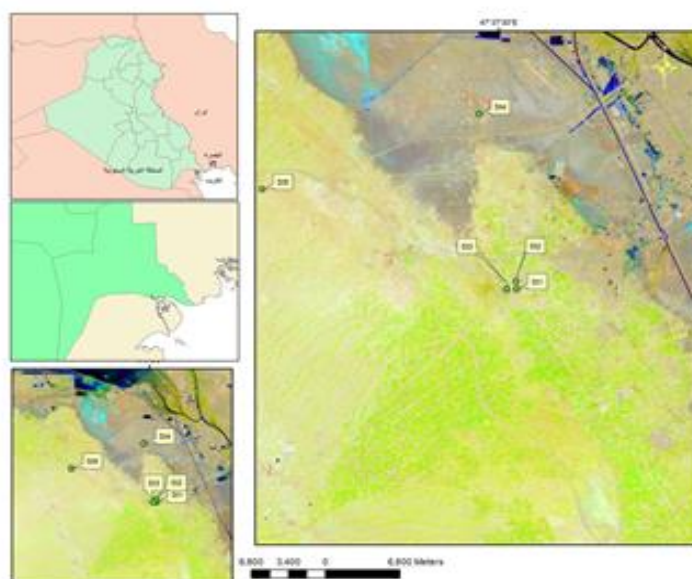


Figure 1. Stations for sampling: 1, 2, 3/ Al-Zubair 4/ Hammar Mushrif gas station 5/ Nahran Omar.

Table 1. Sampling stations coordination.

Sample No.	Latitude	Longitude
1	30.36449	47.63551
2	30.36475	47.63612
3	30.36587	47.63696
4	30.51714	47.60423
5	30.45591	47.39529

### Preparation of media for isolation and purification

The isolation basal salt medium (BSM)<sup>13</sup> has been modified according to Fujii *et al*<sup>14</sup> by

increasing thiamine and biotin, Table 2. The medium components were dissolved in 1000 ml distilled water pH 6.8-7.0 and after sterilization, thiamine, biotin, and 20 ml of methanol was added aseptically to the medium in addition to that fluconazole was added as an antifungal. A 0.5 g of soil was placed in Erlenmeyer flasks containing 100 ml of BSM and incubated for 7 days at 30°C in a shaking incubator (Sartorius, Stidem, Germany) at 180 rpm. Methanol-utilizers were cultured for several replicates on a basal salt medium and were picked up after 5 -7 days of incubation at 30°C.

**Table 2. Composition of medium used for cultivating Methylophilic bacteria.**

Component	Kouno <i>et al</i> <sup>13</sup>	Current study
<i>Nitrogen and Sulfur source</i>		
Yeast extract	0.1 g	1.0 g
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	3.0 g	3.0 g
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.5 g	0.5 g
MnSO <sub>4</sub> .5H <sub>2</sub> O	2.0 mg	2.0 mg
FeSO <sub>4</sub> .7H <sub>2</sub> O	2.0 mg	2.0 mg
<i>Phosphorous source and pH buffering</i>		
KH <sub>2</sub> PO <sub>4</sub>	2.0 g	2.0 g
K <sub>2</sub> HPO <sub>4</sub>	7.0 g	7.0 g
<i>Amino acid</i>		
thiamine. HCl	100 µg	0.2 mg
<i>Vitamin</i>		
Biotin	10 µg	0.1 mg
<i>Antifungal</i>		
Fluconazole	--	0.05 mg

Methanol-salt medium (MSM) of <sup>13</sup> was modified also by increasing the weights of some ingredients and adding fluconazole as an antifungal,

Table 3. The medium was dissolved in 1000 ml distilled water pH 6.8. Fluconazole and 10 ml of methanol were added after sterilization.

**Table 3. Composition of methanol salt medium.**

Component	Kouno <i>et al</i> <sup>13</sup>	Current study
NaNO <sub>3</sub>	2.0 g	-
KNO <sub>3</sub>	-	2.0 g
MgSO <sub>4</sub> .7H <sub>2</sub> O	0.2 g	0.2 g
FeSO <sub>4</sub> .7H <sub>2</sub> O	1.0 mg	10.0 mg
Na <sub>2</sub> HPO <sub>4</sub>	0.21 g	0.21 g
KH <sub>2</sub> PO <sub>4</sub>	0.09 g	0.09 g
CuSO <sub>4</sub> .5H <sub>2</sub> O	50.0 µg	0.5 mg
H <sub>3</sub> BO <sub>3</sub>	10.0 µg	30.0 mg
MnSO <sub>4</sub> .5H <sub>2</sub> O	10.0 µg	2.0 mg
ZnSO <sub>4</sub> .7H <sub>2</sub> O	70.0 µg	10.0 mg
Na <sub>2</sub> MoO <sub>4</sub> . 2H <sub>2</sub> O	10.0 µg	3.0 mg
Fluconazole	-	0.05 mg

### Morphological and Biochemical Tests

The morphological and biochemical tests were used to identify the isolates including to their cell shape, colony morphology, pigment production, Gram staining, catalase and oxidase <sup>15</sup>.

### Extraction of Genomic DNA and Identification of bacteria using 16S rDNA

Bacterial genomic DNA was isolated using the Geneaid Presto™ Mini gDNA Kit (Korea) according to the manufacturer's instructions. A 0.5% agarose gel electrophoresis was used to determine the purity of the DNA. Eluted DNA concentrations were measured using Nano-Drop (Optizen/Korea). 16S rRNA gene sequencing was used to identify consuming methanol bacteria grown on MSM plates, using the following primers: 27F AGAGTTTGATCCTGGCTCAG, 1492R GGTTACCTTGTTACGACTT <sup>16</sup>. A master mix of 25 µl from Go Taq Green master mix (Promega, USA), was mixed with 19 µl of Nuclease Free water, 2 µl (10-20 ng) of DNA template, and 100

Pmol (2 µl) of each primer to a total volume of 50 µl PCR reaction. In order to perform the PCR reaction, the thermal cycler (Eppendorf, Germany) was programmed with the following parameters: 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 Sec, 55 °C for 30 Sec, and 72 °C for 60 Sec, with a final extension at 72 °C for 5 min.

### Gel Electrophoresis

Using a 100bp DNA ladder (Promega, USA) and a UV transilluminator (ATTA, Korea), agarose gel was prepared by dissolving 0.25 g agarose powder in 25 ml TBE buffer with 0.2 g of Ethidium bromide as visualizing dye was used to detect 16S rDNA bands. PCR amplicons were sent to MacroGen for further purification and sequencing. The National Center for Biotechnology Information's BLAST was employed to align sequences in order to identify the isolated methylophilic through 16S rRNA gene.

[https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE\\_TYP E=BlastSearch](https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYP E=BlastSearch)

#### Identification of the isolates through *MxaF* gene

The methylotrophic isolates were identified using *MxaF* specific primers which encodes for gene methanol dehydrogenase enzyme, producing an amplicon of ~ 550bp. The primers sequences were:

F1003degen 5'-GGNCANACYTGGGGNTGGT-3', R1561degen 5'-GGGARCCNTTYATGCTNCCN-3'<sup>17</sup>.

Each PCR reaction mixture included 12.5 µl of Go Taq Green master mix (Promega, USA), 7.5µl of Nuclease Free water, 3 µl (15-30 ng) of DNA template, and 1 µl (100 Pmol) of each primer make up a 25µl PCR reaction mixture. The thermal cycle was set (Eppendorf, Germany) as the following conditions were used for the PCR reaction: 94 °C for 45 Sec, 59 °C for 1 min, and 72 °C for 1.5 min, for 30 cycles, with a final extension at 72 °C for 10 min.

#### Biodegradation of crude oil

Pure cultures of the isolated bacteria were prepared by adding 1 ml of the liquid pure culture in

a conical flask containing 100 ml of MSM and 0.5% (v/v) crude oil supplied from Al-Shua'aba Refinery-Basra city. The flasks were incubated in a shaking incubator for 7 days at 30 °C with 120 rpm<sup>18</sup>.

#### Extracting residual crude oil

A liquid-liquid extraction technique was used to extract the leftover crude oil by a separating funnel. The aqueous phase was discarded, and the remaining oil was dried in the oven at 40 °C to remove the chloroform. The aliphatic fraction was separated, and the residual oil was diluted in 25 ml of n-hexane. The aliphatic fraction was collected and sent to be analyzed by Gas Chromatography to estimate aliphatic compounds (Agilent Chem Station)<sup>19</sup>.

#### Results:

##### Isolation of Methanol utilizers

Little pink spherical colonies began to form after 3–5 days of incubation at 30 °C. gram-negative bacteria might be seen alone, in pairs, or in large numbers. There was no spore formation, and tests for catalase and oxidase were positive, Fig. 2.

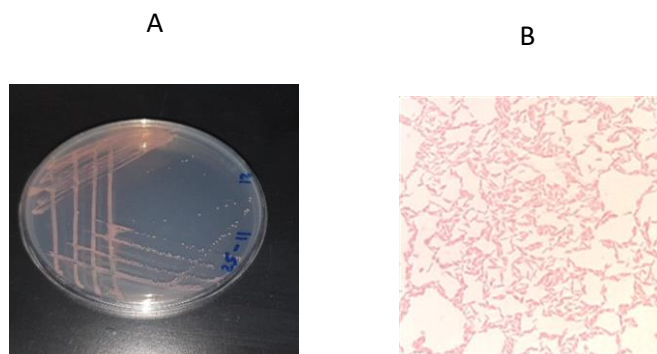
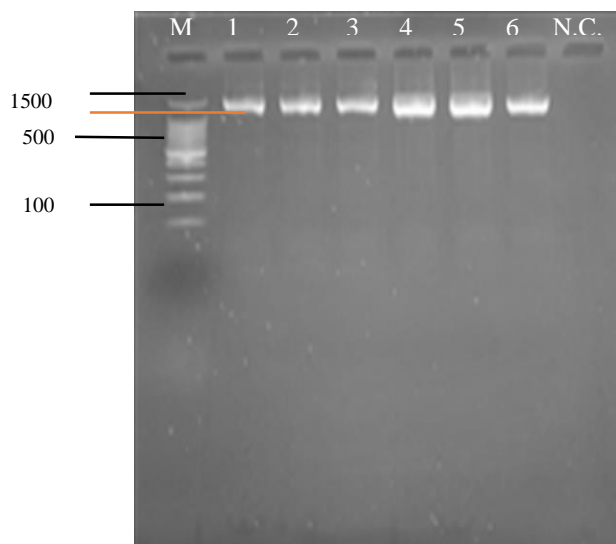


Figure 2. A/ *M. extorquens* colonies in MSM agar. B/ cells of *M. extorquens* under microscope x 100.

#### Identification of the isolates through 16S rRNA gene

The sequencing of six nominated isolates revealed the presence of methylotrophic bacteria using 16S rRNA amplicon size of 1500bp on a 1%

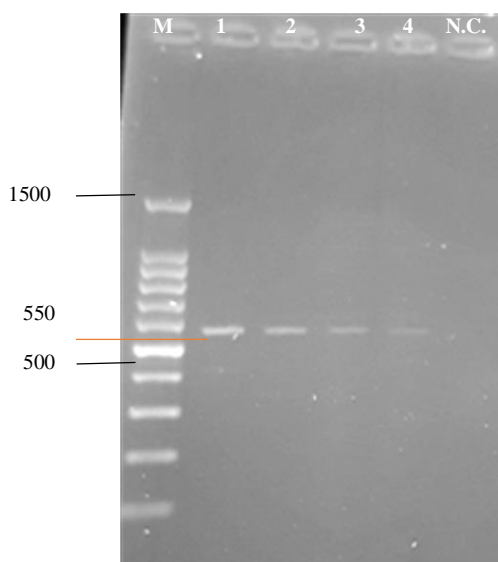
agarose gel. *Methylorubrum extorquens* were identified at the species level based on a 99% similarity of 16S rRNA sequences to the intended type in GenBank Fig. 3.



**Figure 3. Amplification of the 16S rDNA gene (~1500bp) on an agarose gel electrophoresis at 60 volts and 1 hour. Lane M: 100bp Marker, Lane 1-6: 16S rDNA gene bands for isolates No. 1 to 6, Lane 7: Negative control.**

#### Identification of bacteria using *MxaF* gene

Using the *MxaF*- specific primers, *MxaF* was detected in four isolates at the conservative region of methylotrophic bacteria, the isolates were genetically identified. On a 2 % agarose gel, the *MxaF* amplicon names and affiliations of *Methylobacterium* genomic DNA were visualized at the expected size of 550bp, Fig. 4.



**Figure 4. The amplified *MxaF* gene (550bp) on an agarose gel electrophoresis at 60 volts and 1 hour. Lane M: 100-bp marker, Lanes 1-4: *Methylobacterium* sp. *MxaF* gene bands NC: Negative Control.**

#### Biodegradation of crude oil

Significant increases in cell density were seen after 7 days of incubation when crude oil was used as the sole carbon and energy source, with simultaneous decreases in several components of the used crude oil, Fig. 5. During the incubation time, there were significant changes in numerous components of the used crude oil. After 7 days, most of the peaks had significantly shrunk. The short-chain alkanes  $nC_{12}$ - $nC_{15}$  were efficiently destroyed by presumptive *M. extorquens* which the best-growing isolate was used on crude oil, while long-chain alkanes  $nC_{16}$ - $nC_{37}$  degraded at a slower rate. The total degradation ratio was 61.14% (0.5% v/v) of crude oil, Fig. 6.

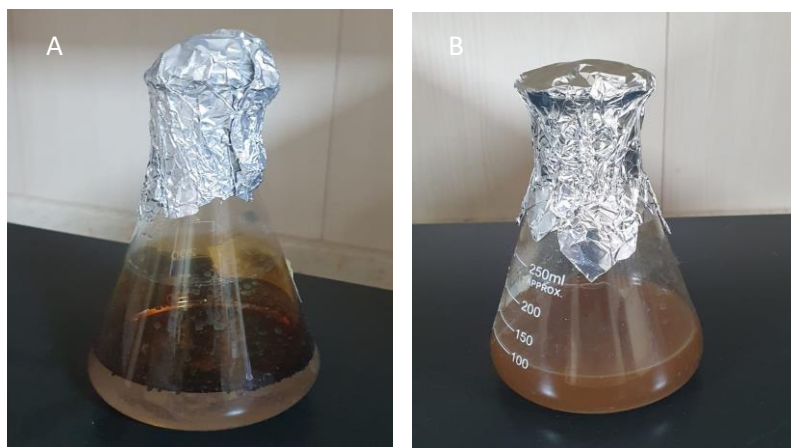


Figure 5. Crude oil degradation by *M. extorquens* after incubated at 30 °C with 120 rpm shaking. A/ control has crude oil only. B/ bacteria with crude oil after 7days' of incubation.

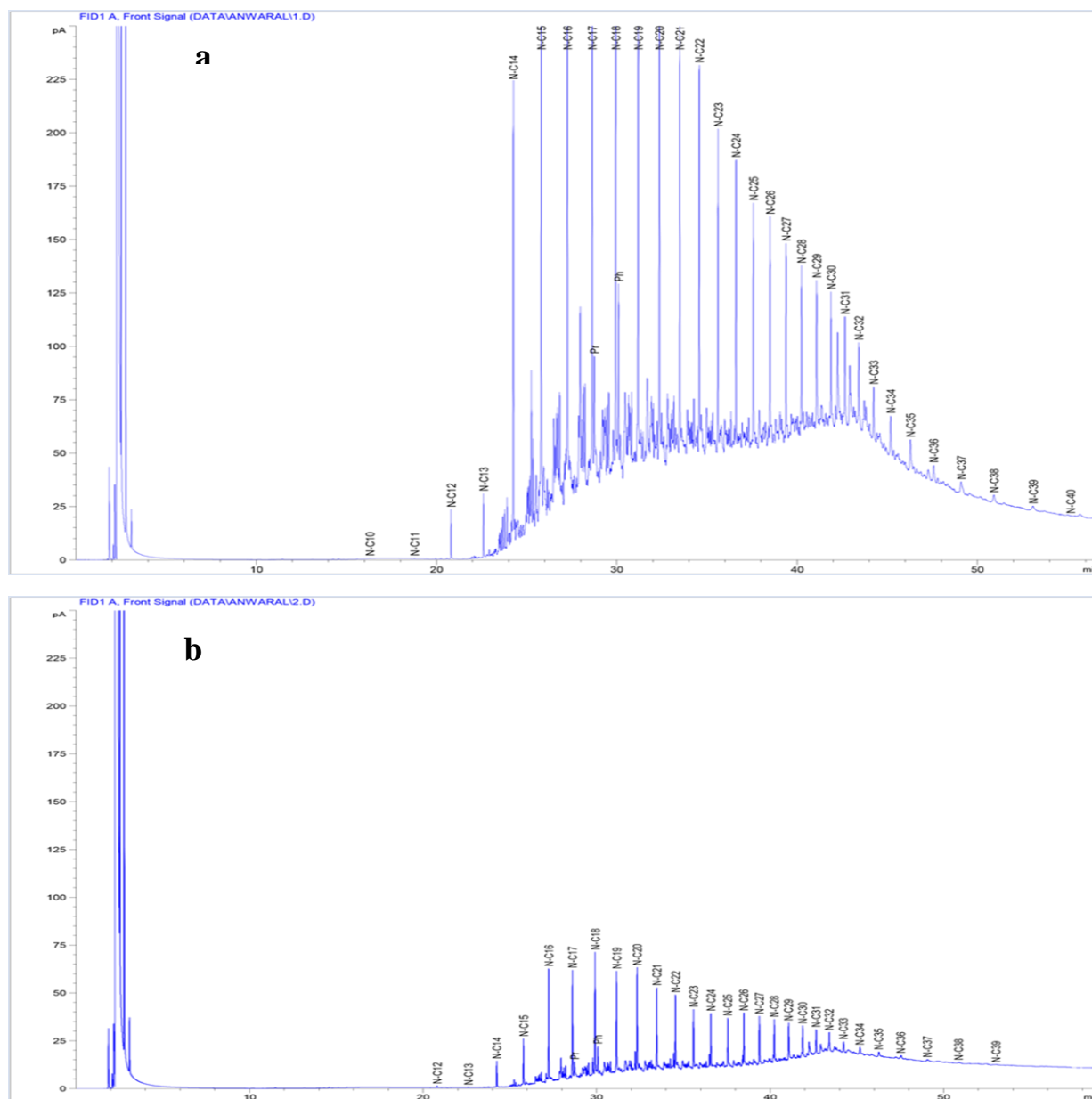


Figure 6. GC chromatograms of aliphatic compound following crude oil degradation (a) crude oil in the control group; (b) aliphatic compound following biodegradation.



## Discussion:

The study aimed to isolate and identify facultative methylotrophs from oil contaminated sites in Basra- Iraq and test their ability to naturally degrade the crude oil at the laboratory.

In this study, a new modified medium has been introduced according to the ability of bacteria to grow. The isolates grew very poorly on the methanol-salt medium and did not grow if the inoculum was small. Also, the isolates could not grow in the medium of Kouno *et al*<sup>13</sup>. This may be due to the harsh environment from which they were isolated. So, the increase of biotin and thiamine and the addition of antifungals led to the flourishing of these bacteria and increased their ability to consume methanol, which reached 6% (unpublished data) and reduced the incubation period.

The results of morphologic and biochemical tests of *Methylobacterium* according to Bergey's Manual of Determinative Microbiology's description of phenotypic identification of *Methylobacterium* species<sup>20</sup>. These bacteria were genetically identified using the 16S rRNA gene as *Methylorubrum extorquens*. These results come in consistent with findings published by Rojas-Gätjens *et al*<sup>21</sup> who detected an abundance of methylotrophic bacteria in oil contaminated areas, including *Paracoccus communis* and *Methylorubrum rhodesianum*. Furthermore, Godini *et al*<sup>22</sup> isolated nine bacterial species from oil-polluted locations from Iran's Kharg Island; *Methylobacterium persicinum* was one of them.

In the current study, we found that methanol can be consumed during colonization by *Methylorubrum* in addition to using carbon sources other than methanol, such as crude oil. This is consistent with the study conducted by Hu and Lidstrom<sup>23</sup>, which found that methylotrophs can consume single-carbon compounds or multiple-carbon compounds without a carbon-carbon bond.

The *MxaF* gene has been used to detect methylotrophs in the environment because it is highly conserved among the methylotrophs that have been studied<sup>24</sup>. A pair of specific primers was chosen from the common conserved area of the *MxaF* gene for the identification of methylotrophic bacteria, to validate the identification of the genus *Methylorubrum* and to detect the *MxaF* gene. The size was amplified by these primers, resulting in a 550-bp amplicon. The *MxaF* gene was employed by Lau *et al*<sup>16</sup> as a biomarker for methanotrophic proteobacteria found in the *Methylocystaceae* and *Methylococcaceae* families.

In contaminated environments, petroleum hydrocarbons are digested by bacteria and used as their sole source of carbon and energy. Genetics

determines a microbe's ability to incorporate molecular oxygen into a hydrocarbon and generate intermediates that enter the cell's overall energy-producing metabolic pathway<sup>25</sup>.

A new strain of *Methylorubrum extorquens* has been isolated and with the ability to exploit the aliphatic hydrocarbon compounds as a source of energy in addition to methanol, in spite of carbon-carbon bonds. This is a globally new finding. So, after 7 days of incubation, the rate of degradation was 61.14% and short-chain alkanes nC<sub>12</sub>-nC<sub>15</sub> were broken down, whereas long-chain alkanes nC<sub>16</sub>-nC<sub>37</sub> degraded at a slower rate. Moreover, these bacteria have the ability to degrade aromatic hydrocarbon compounds (unpublished data). Perhaps the reason behind that, is these bacteria have been isolated from harsh environments and exposed to many types of oil and chemical pollutants, in addition to high temperatures that may sometimes reach more than 65°C, forcing them to adapt to these conditions and exploit what is available to them from nutrients.

This degradation rate is less than the 83.8 and 81.63% at 0.5 % crude oil reported for *Vibrio vulnificus* and *Brevundimonas diminuta* respectively and higher than 51.64 and 58.31 % at 7 days reported for *Ochrobactrum anthropic* and *Sphingomonas paucimobilis* isolated from contaminated soils in the Khor Al-Zubair channel, southern Iraq<sup>18</sup>.

As a source of carbon and energy, the bacteria used the hydrocarbon substrate, as evidenced by a considerable reduction in peaks between 0 to 7 days which coincided with the exponential development of bacteria. Salam *et al.*<sup>26</sup> also found that *Methylobacterium mesophilicum* strain RD1 destroyed 61.2 and 89.5 % of the starting concentration of the used motor oil during 12- 21 days, respectively. They explain that this is because strain RD1 contains numerous degradative genes.

## Conclusion:

Due to the high oil pollution at the locations of *M. extorquens* isolation, these sites are of significant interest for isolating novel hydrocarbon-degrading bacteria with high catabolic abilities enhanced by living in a highly polluted site. Without no doubt, this elevated bioremediation ability is attributed to the high and continuous exposure to hydrocarbon chemicals over time. In the present study, conventional PCR was used to identify methylotrophic bacteria and the methanol dehydrogenase enzyme. The analysis of GC showed about 61.14% of crude oil was degraded, and the GC fingerprinting appears to show that C<sub>12</sub>, C<sub>13</sub>, C<sub>14</sub>, and C<sub>15</sub> had decreased significantly. Our

findings of the ability of *M. extorquens* to successfully metabolize aliphatic compounds can be employed and genetically upregulated to bioremediate aromatic compounds in other studies. *M. extorquens*' ability to break down crude oil is a good start for genetically modifying these strains to make them twice as good using advanced methods like CRISPR Cas9.

#### Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in University of Basra.

#### Authors' Contribution Statement:

The authorship have participated in different roles as follows:

The research plan was developed by A A-T and Z A as supervisors for the PhD student A A. M. The research was conducted and written by the student, and it was reviewed and revised by her supervisors.

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## التشخيص الجزيئي لبكتريا *Methylorubrum extorquens* باستخدام أجزاء جين *MxaF* المضخمة بال PCR كعلامة جزيئية

زينة وحيد عطوان<sup>2</sup>

أسعد محمد رضا الطائي<sup>1</sup>

أنوار عبدالوهاب مكي<sup>1</sup>

<sup>1</sup>قسم التطور الاحيائي، مركز علوم البحار، جامعة البصرة، البصرة، العراق.  
<sup>2</sup>قسم الاحياء المجهرية، كلية الطب، جامعة البصرة، البصرة، العراق.

### الخلاصة:

بكتريا methylotroph واسعة الانتشار ولها القدرة على استهلاك كربون واحد (C1) مما يجعلها آلات تحويل بيولوجي. هي الدراسة الأولى التي تبحث عن بكتيريا methylotrophic الاختيارية في التربة الملوثة في العراق. تم استخدام PCR التقليدي لتضخيم *MxaF* الذي يشفر إنزيم Methanol dehydrogenase. تم استخراج قوالب الحمض النووي من البكتيريا المعزولة من خمسة مواقع ملوثة في البصرة. اكتشف تفاعل PCR باستخدام جين مخصص أن *Methylorubrum extorquens* هي الأنواع الأكثر انتشاراً في هذه البيئات. تم اختبار قدرة *M. extorquens* على تحلل مركبات الهيدروكربونات الأليفاتية في المختبر. في غضون 7 أيام، كشفت دراسات Gas Chromatography لبقية النفط الخام المستخدم أن 61.14% من المحتوى الأولي قد تكسر، وأظهرت بصمة GC للمركبات الأليفاتية المستخدمة انخفاضات كبيرة في C<sub>12</sub>، C<sub>13</sub>، C<sub>14</sub>، C<sub>15</sub>، هذه هي المرة الأولى التي يتم فيها العثور على سلالة جديدة من *M. extorquens* على مستوى العالم لديها القدرة على تحلل مركبات الهيدروكربونات الأليفاتية. أوضح استخدام جهاز PCR التقليدي والتسلسل الجيني عن وجود بكتيريا methylotrophic في المناطق الملوثة في البصرة. كانت *M. extorquens* هي السائدة وأظهرت قدرة كبيرة على تحلل النفط الخام مما يجعلها أداة مهمة لاستخدامها في المعالجة الحيوية.

**الكلمات المفتاحية:** جهاز كروماتوغرافيا الغاز، انزيم الميثانول ديهدروجينيز، جنس الميثيلوربرم اكتروكس، بكتريا الميثيلوتروف، جين *MxaF*