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Efficiency of *pseudomonas fluorescens* in the dissociation of hydrocarbons and statement their molecular characterization of biodegradable

Baidaa Hussain Jassim

Baidaa.hussin@mu.edu.iq

Department of Pathological analytics, College of Applied Medical Sciences, University of Al-Muthanna, , Iraq.

Abstract:

The study investigated the efficiency of *pseudomonas fluorescens* bacteria in the degradation and consumption of hydrocarbons, isolating 15 isolates of crude oil from different environmental zones, including the (soil of the generators, the soil of the oil change shop, the soil of the diwaniya rubber plant,(Which were contaminated with hydrocarbon compounds) and Drainage water). A number of variables were used to distinguish the ability of bacterial isolates to analyze hydrocarbon compounds by their ability to grow on the medium of solid mineral salts and the addition of crude oil. The bacterial isolates During the period of incubation for a maximum of 7 days and at a temperature of 37 ° C of consumption of crude oil as a single source of carbon and energy in addition to its growth on the center of liquid mineral salts with crude oil by 1% The electrical conductivity and optical density were measured. Electrical conductivity and optical density were continuously measured to indicate the activity of bacterial isolates in decomposing hydrocarbon compounds while in a mineral salt medium supplemented with 1% crude oil. An increase in both electrical conductivity (EC) and optical density (OD)

was observed in all bacterial isolates during the incubation period, indicating their efficiency in hydrocarbon degradation. P. fluorescens isolates from generator soil were the most active, exhibiting the highest electrical conductivity (12.7 mS/cm) and an optical density of 0.322.

The enzyme responsible for hydrocarbon degradation, catechol 2,3-deoxygenase, was identified, with the gene appearing in 8 bacterial isolates (53.33%). Sequence analysis of the enzyme in P. fluorescens showed a 100% match with the gene registered under number KF010862.1.

KEYWORDS: phenotypic *P. fluorescens* , $C_{23}O$ gene, Catechol enzyme 2,3 dioxygenase .

Introduction:

Hydrocarbons such as diesel, crude oil, lubricants and distillates are the most widely used sources of energy and fuel (1)., The Iraqi environment is exposed to hydrocarbon pollution from various sources. mainly oil refineries and power plants that operate in black oil and result from soil pollution with hydrocarbons change in many physical, chemical and biological properties of soil, which negatively affects agricultural production as well as its effect on soils (2). Biological treatment involves the use of microorganisms to decompose hydrocarbons And the use of carbon monoxide as a sole source of carbon and energy leading to the decomposition of contaminants in full and thus reduce the proportion of substances with toxic and carcinogenic effect as well as reduce the proportion of organic carbon (3) in addition to the low cost of biological treatment and is accompanied by environmental disturbance and lack of contact between the workers (4). The bioremediation process involves two directions, one of which is an increase in the degradation rate of the organisms by creating suitable environmental conditions for their growth from heat, pH and ventilation, and the other depends on the development of genetically modified germ strains that have metabolic capacity to be analyzed in the contaminated areas (5). The rate of occurrence of biological decomposition is associated with several factors, the chemical composition of the pollutants, the optimum environmental conditions that stimulate the effectiveness of

biodegradation, interactions between the number and type of organisms, the persistence of hydrocarbons in the soils (6). Bacteria are microorganisms eligible for bioremediation of oil residues as they destroy organic and hydrocarbon substances and use them as a sole source of carbon and energy by producing a large group of soluble enzymes as well as their adsorption to many (7). This study focused on isolating and diagnosing *pseudomonas fluorescens* bacteria that are effective in analyzing complex compounds and conducting molecular studies to try to determine the physiological and genetic basis that controls the efficiency of analysis and consumption of these complex.

Materials and methods:

Collecting, isolating and diagnosing bacterial isolates: During the study, 200 samples were collected from different sources in the city of AL Diwaniyah, including the soil of the generators, oil change shop, the soil of the rubber plant, Drainage water (table 1). (fecundate 1_g of soil samples and 1_{ml} of water samples in 50_{ml} of mineral salts containing 4_g of NH4CL, 0.2_g of $7H_2OMgSO_4$, 0.1_g of NaCl, 1.8_g of K_2HPO_4, 0.01_g of $NaSO_4.7H_20$) as medium Crude oil by 1% as a source of energy and carbon then incubated in a hatching incubator at 180 $_{rpm}$ and at a temperature of 37º C for 7 days (8).

Dissolve 0.1_{ml} of the mineral salts in which samples were planted and show bacterial growth on (Stromide agar, macconkey agar , and blood agar) groups after applying appropriate scavenging using the phosphate solution and incubating the dishes at 37° C for 24 $_h$ after which the colonies with the traits General of *P. fluorescens* (8). Studied the phenotypic properties of isolated bacterial colonies and microscopic properties of cells. Biochemical tests were performed and the diagnosis of (API 20E) to confirm the accuracy of bacterial isolates (9).

Sample collection site	number
soil of the generators	50
the soil of the oil change shop	50
the soil of the diwaniya rubber plant	50
Drainage water	50
Total	200

Table (1) showing the number and Sample collection site

Testing the susceptibility of bacterial isolates to the dismantling of hydrocarbons:

Several variables were used to distinguish the ability of bacterial isolates to decompose hydrocarbons by measuring both optical density and electrical conductivity (10).

Use the optical spectrometer to measure growth in terms of optical density and a wavelength of (600_{nm}). The changes in the electrical conductivity of the bacterial pollinated medium were measured in (milli Siemens / cm) units by used Conductivity Meter .

The ability of the bacterial isolates to decompose the hydrocarbon compounds in the steel medium and their consumption as a sole source of carbon and energy was studied: The concentrated dishes of the mineral salts added 1% crude oil with the bacterial isolates vaccine and incubated the dishes for seven days at 37 ° C.

- **Molecular properties: - Genomic DNA is isolated from bacteria:** DNA was extracted from bacterial isolates by transferring $1_{\mathbf{Mm}}$ of bacterial implants using the Wizard Genomic Extraction Kit produced by (Promega-USA) according to the manufacturer's instructions.

Detection of Catechol 2,3dioxygenase ($C_{23}O$ gene): $C_{23}O$ gene was amplified by using the primer pair of the forward primer (CGAACGATTCATGACCGTGC) and the reverse primer (TTCCAGGTCATGAGCAGCAG). The DNA amplification was performed as the final volume of the DNA amplification product was (DNA 20 µl, 1µl of 10_{pmol}) per for each forward primer and reverse (2.5µl of the DNA template, 15.5µl of PCR water at 1_x concentration), use a Thermocycler PCR device for serial polymerization reaction. The reaction steps were followed by an initial phase of 95 ° C initial denaturation for 2 minutes followed by 30 cycles of amplification (95 ° C For 30 seconds, 59.3ºC for 30 seconds, 72ºC for one minute) finally the final extension phase of 72ºC for 5 minutes.

Sequential sequencing analysis of the encoded gene of the catechol enzyme 2,3 dioxygenase:

The test was performed after obtaining the results of the PCR reaction of the catechol 2,3 dioxygenase of the *P. fluorescens* isolates. The PCR reaction product was sent to Macrogen in South Korea to serialize the DNA by using the AB DNA sequencing system(21). After

sequencing the gene bases were analyzed using the NCBI-BLAST identifier site to identify genetic mutations and phylogenetic tree analysis using the MEGA 6.0 program to demonstrate the genetic variation between isolates and standard isolation. Finally, isolates were recorded at the NCBI-Genbank Submission .

Results and discussion:

Diagnosis of bacterial isolates: isolates isolated *P. fluorescens* isolates from four different environmental zones based on their physiological, agricultural and biochemical characteristics. Table (2) In addition to diagnosis using the ($API_{20}E$) system (Fig 1). The bacteria were Gram-negative because their cell wall consists of a thin layer of peptidoglycan, which appears pink. They were positive for the oxidase test, indicating the presence of an cytochrome oxidase enzyme used in aerobic respiration, and positive for the catalase test, indicating the presence of the catalase enzyme, which breaks down hydrogen peroxide into water and oxygen to protect the cell . They were positive for the motility test because the bacteria are motile due to the presence of polar flagella (22). They were negative for the indole test because they do not contain the tryptophanase enzyme, which breaks down tryptophan to produce indole, and negative for the methyl red test because they do not ferment glucose. They were also negative for the voges-proskauerl test because they do not produce acetone from glucose fermentation. However, they were positive for the citrate consumption test because they can use citrate as a source of carbon and energy. Negative for the urease test as it does not contain the urease enzyme, which breaks down urea into ammonia. Negative for the starch hydrolysis test as it lacks the amylase enzyme. Negative for the glucose fermentation test as it does not ferment sugars and relies on aerobic respiration (23).

(Fig 1) Diagnosis of $API_{20}E$ for *P. fluorescens* bacteria

Test bacteria	Gram stain	oxidase	catalase	The movement	urease	Test IMVC			
						C	VP	MR	I
P. fluorescens	–	+	+	+	+	+	–	–	–

Table (2) Biochemical tests (+) positive result of the examination, (-) Negative result of the examination

-Test the ability of bacterial isolates to dismantle hydrocarbons:

1- Growth on the medium of liquid mineral salts: The results showed, as shown in Figure (2), the ability of bacterial isolates to grow on the medium of liquid mineral salts added to the crude oil and crude oil consumption during a period of 6 weeks of incubation. The isolates consumed all crude oil with the survival of the control flasks unchanged and the ability of bacterial isolates to grow on the liquid saline medium and its activity in the degradation of hydrocarbons to Emulsifying agents. When bacteria consume hydrocarbons, this is often associated with the secretion of these bacteria to the environment or the

food medium that grows on them. These substances reduce the surface tension and increase the penetration and spread of water, which leads to the growth of bacteria and the retention of minerals and separation. The emulsification makes the oil layer in the form of oil droplets emulsion and thus increase their solubility and easily blend with the components of the saline medium while providing a surface area suitable for microbiological contact with these compounds. This is confirmed by several studies, including the study of (11,12).

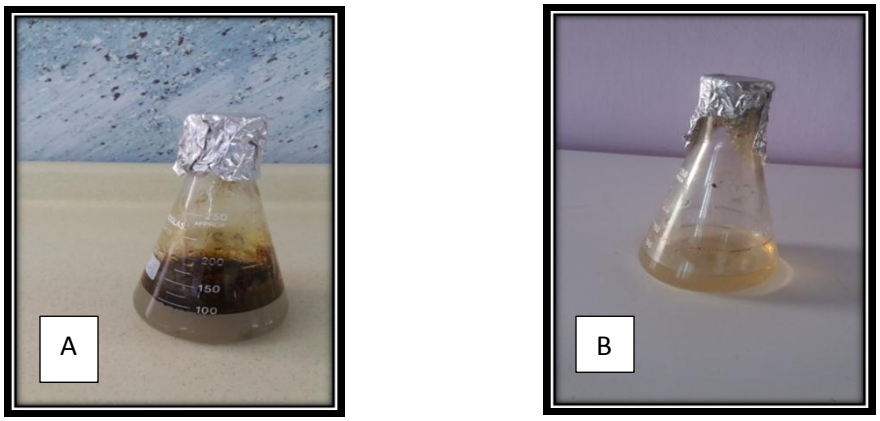


Figure (2) Consumption of crude oil by *P. fluorescens* bacteria on the medium of liquid mineral salts (A): negative (B): positive

2- Growth on the medium of the mineral salts steel: The results showed in Fig (3) the ability of the bacterial isolates of *P. fluorescens* bacteria to decompose the crude oil on the medium of the mineral salts steel. The bacterial isolates during the incubation period of up to 7 days of consumption of crude oil as a source this result is similar to that of (13).

The ability of these isolates to disassemble hydrocarbons is due to the presence of certain enzymes that conduct hydrocarbon analysis, such as Catechol 2,3 dioxygenase (14) or have been under These isolates on plasmids are often metabolic plasmids with large molecular weights that encode the process of biodegradation and are responsible for the molecular organization of the process (15).

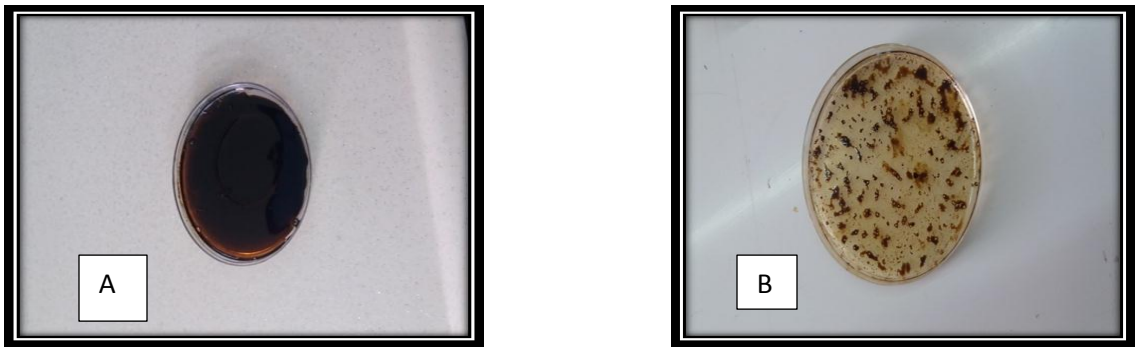


Figure (3) Consumption of hydrocarbons by *P. fluorescens* bacteria on the medium of the hard mineral salts (A):negative (B):positive

- Estimation of growth in terms of electrical conductivity:

The results of the electrical conductivity calculation showed an increase in the electrical conductivity values of all bacterial isolates during the incubation period compared to the control with the electrical conductivity values of 7.2 mc / cm. The growth rate gradually increased until it reached the maximum in the third week of incubation. With a gradual transfer to reach the lowest value in the sixth week and recorded *P. fluorescens* isolated from generated soil the highest electrical conductivity value (Fig.4). The increase in electrical conductivity values indicates a direct relationship between conductivity and ion concentration and valence. Conductivity values increase with increasing ion concentration. An increase in ions in the third week means that the gene encodes the catechol enzyme, which has broken down hydrocarbon compounds and released ions into the culture medium. Furthermore, the accumulation of metabolic products in the medium contributes to increased conductivity. Consumption of petroleum hydrocarbons by bacterial isolates leads to an increase in these products. Electrical conductivity depends on the metabolic products formed and their ionization capacity in water, resulting in increased conductivity values. This study is consistent with (16).

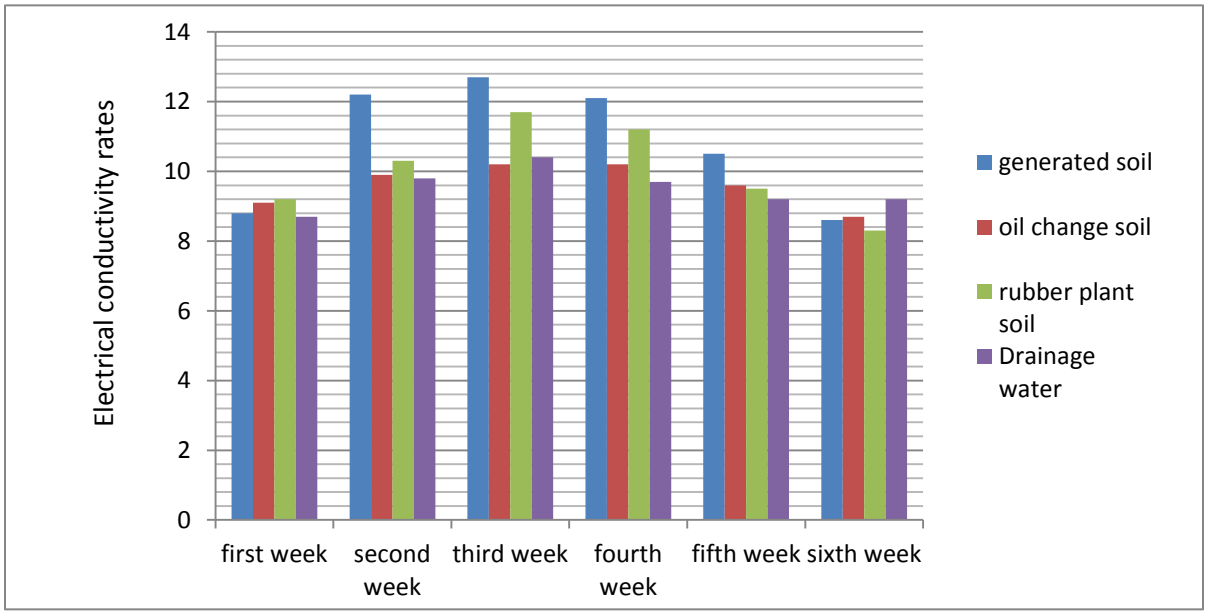


Figure (4) Growth rates in terms of electrical conductivity within six weeks of incubation

- **Determination of growth in terms of optical density:**

The results showed that the isolates were able to grow on the medium of mineral salts with the crude oil used in this study. It was noticed that during the incubation period there was a gradual increase in growth. These results were agreed with (17) who indicated that when consuming bacteria for hydrocarbons, Gradually, the rate of growth gradually increased to reach the highest value in the fourth week of incubation. After the fourth week, the intensity of the optical intensity gradually decreased to reach the lowest value in the sixth week. Isolates *P. fluorescens* isolated from generated soil the highest density of light intensity (fig.5) (18) reported that bacteria use bacteria to use simple hydrocarbons first and then consume the most complex complex compounds. If the bacteria are consumed only for simple hydrocarbons, cell counts increase rapidly and then begin to decrease. In case bacteria consume complex compounds, However, cell counts continue to increase or stabilize relatively.

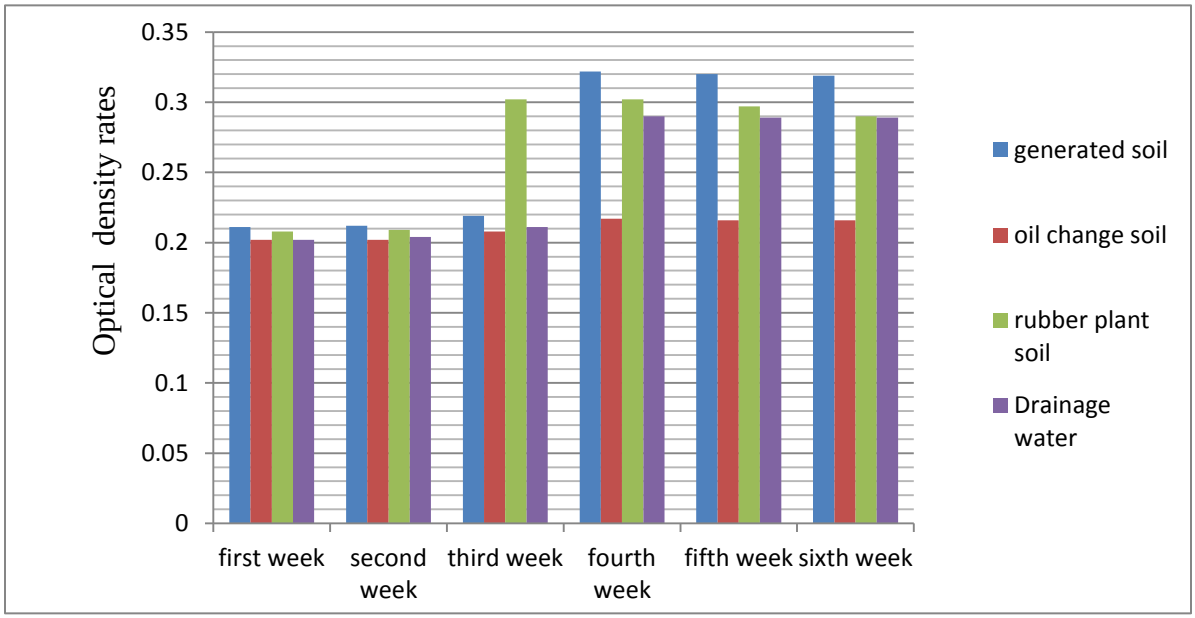


Figure (5) Growth rates in terms of light density during six weeks of incubation

- Detection of the encoded gene for Catechol 2,3dioxygenase ($C_{23}O$)

Fig (6) shows that the containment of *P. fluorescens* isolates on the ($C_{23}O$) gene, which encodes the production of Catechol 2,3 dioxygenase. This enzyme plays a significant role in the degradiation of hydrocarbons (19). The presence of Catechol 2, 3dioxygenase using a $C_{13}O$ initiator to give a PCR (553bp) output as in Fig (6).

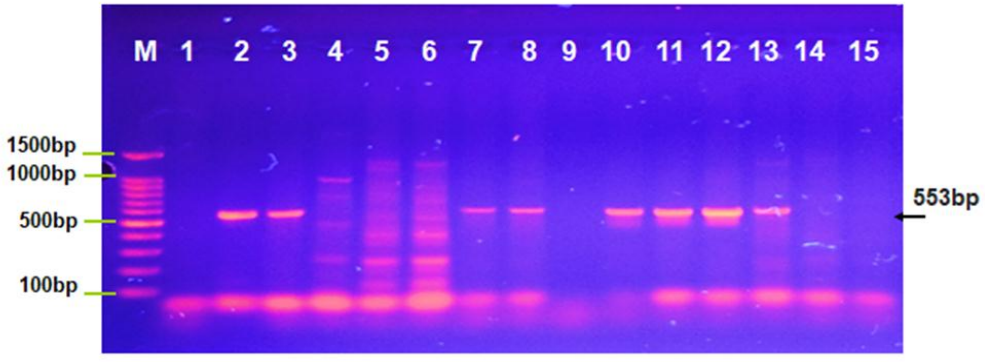


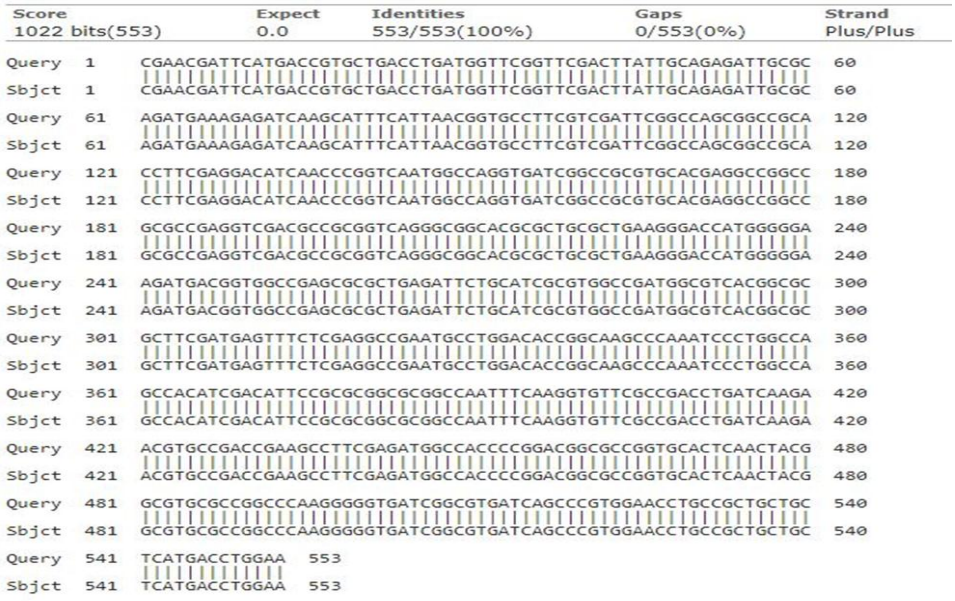
Figure 6: Electroporation using agarose gel showing the results of the PCR test to investigate the catechol 2,3 dioxygenase in *pseudomonas fluorescens* where M:

Marker (1500-100 pb) and numbers (23,22,20,12,8,7) represent positive isolates of the Catechol gene 2,3 dioxygenase with a resultant its length of (553pb).

Results showed that the number of *P. fluorescens* isolates of the enzyme was (8) by 53.33% and agreed with the results of (20).

- **Sequential Sequencing Analysis Technique for the Catechol Enzyme Enzyme 2,3 dioxygenase:**

Fig (7) shows sequential sequencing of the $C_{23}O$ encoding gene of Catechol 2,3dioxygenase in comparison with standard isolation. Sequencing of Gen ($C_{23}O$) in isolates of *P. fluorescens* was analyzed using the MEGA6 program and compared to the sequential sequencing of the original gene of standard isolation and showed, the results are 100% identical with the standard isolation gene carrying the serial number (KF010862.1) .



(Fig 7) represents a multiple sequencing alignment analysis using the MEGA6 program for gene catechol 2,3 dioxygenase in *pseudomonas fluorescens*.

Conclusions:

1- The ability of the bacterial isolates identified in this study, from different sources of isolation, to break down and consume petroleum hydrocarbon compounds indicates that these bacteria can be used in the bioremediation of petroleum hydrocarbon compounds.
2- Environmental pollution in certain areas leads to the presence of bacterial species capable of decomposing petroleum hydrocarbons. These organisms adapt to consuming these pollutants and using them as a source of carbon and energy, thus mitigating the severity of environmental pollution.
3- Molecular investigation of the (C23O gene), which encodes the production of the catechol 2,3 dioxgenase enzyme, confirmed the presence of this enzyme in 8 bacterial isolates, representing 53.33%.

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