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Research Article

Molecular Analysis of *phe* Operon Genes determining Phenol-Degrading *Pseudomonas* sp. from Polluted Sites in Baghdad City

Huda Rasheed Tawfeeq*, Sawsan Sajid Al-Jubori, and Amel Hussaein Mussa

Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq

Abstract: Phenolic compounds are toxic to plants, animals and even for microorganisms at low concentrations. Because of this toxicity, it is important that soils polluted with these compounds to be remediated immediately. *Pseudomonas aeruginosa* and *Pseudomonas putida*, as well as their both intra- and extradiol enzymes, were the targets of this study, which aimed to detect the enzymes responsible for phenol degradation capability in bacteria and the genetic variation of the catabolic genes related to the phe operon among the positive isolates. In this study one hundred twenty five samples of contaminated soils have been collected from different sources at Baghdad city (89 samples from Daura refinery, 21 samples from private electricity generators and 15 samples from different farm lands). Collected samples have cultured on mineral salt medium as well as using differential and selective media, then diagnosed by classical biochemical tests and VITEK system beside using Housekeeping gene 16s rDNA for molecular diagnosis. The results of VITEK system revealed that 29 /89 (32.5 %) of samples from Daura refinery had *P. aeroginosa* isolates and only one sample 1/89 (1.1 %) of *P. putida*. On the other hand, none of the samples from generators (0 %) were *P. aeroginosa* and 5/21(23.8 %) were *P. putida* while 5/15 (33.3 %) samples of farm lands were *P. aeroginosa* and (0 %) were *P. putida*. Molecular diagnosis using 16S rDNA detected 40/125 (32 %) positive isolates for *Pseudomonas* sp.; 34 (85 %) isolates for *P. aeruginosa* and 6 (15 %) isolates for *P. putida*. Phenol degradation capability of the forty isolates has been tested on mineral salt medium using different concentrations of phenol (100 ppm to 1500 ppm) and all of them (100 %) were able to degrade phenol to 600 ppm but a number of 4 isolates (10 %) have exceeded this concentration to 1200 ppm and only one isolate (2.5 %) tolerated phenol to the maximum level which is 1500 ppm. Phenol degrading isolates were subjected to PCR technique to detect the phe-like genes: catechol 1, 2 dioxygenase (*cat*1), and catechol 2, 3 dioxygenase (*cat*2). As a result, this set of enzymes were found in the whole five (12.5 %) isolates that effectively degraded phenol to the concentration of 1200 ppm and 1500 ppm.

Keywords: Pollution, Phenol, Phenol-Degradation, Biodegradation, *phe* genes, *Pseudomonas aeruginosa*, *Pseudomonas putida*, Catechol Dioxygenase.

1. INTRODUCTION

Toxic environmental pollutants such as phenol originate primarily from industrial processes and it has been listed among the most common pollutants. In order to protect the environment, the U.S. Environmental Protection Agency has set a limit of 0.1 mg/L of phenol in the water supply. Removal from the environment is necessary. For the treatment of phenol-contaminated wastewater, more effective and less expensive biodegradation methods are available. More and more microbes have been discovered to coexist in almost all natural environments in the last three decades, particularly in soils, where microbial catalysts have been used extensively [1]. Oil production stations and refineries serve as the major contributors in the environmental problems especially in soil and water [2]. Oily wastewater and soils contains hazardous and toxic substances that have inhibitory effects on animal and plant growth [3] as well as their mutagenic and carcinogenic effects on humans around the world [4, 5].

Numerous studies suggested that biodegradation has become a priority for scientific

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*Corresponding Author: Huda Rasheed Tawfeeq <hudar2@yahoo.com>

research as it aims to safely and quickly remove for these contaminants from soils [6] and confirmed that the final success of biodegradation depends on the nature of microorganisms which are in direct contact with the biodegradable substance, and that the bio-treatment of soils contaminated with hydrocarbons from crude oil linked to the ability of organisms to consume hydrocarbons [7].

Treatment of oil- contaminated soils is done by the aid of common microorganisms or ones isolated from oil- contaminated sites. The ability of these microorganisms for biodegradation was tested *in vitro* [8]. Many types of bacteria have been isolated from contaminated soil and one of these are *Pseudomonas* spp. These microorganisms gradually reduced the concentrations of polycyclic hydrocarbon compounds due to their ability to survive in such soils by developing a certain enzymatic and physiological response allowing them to use hydrocarbonic compounds present in oil as an alternative source of carbon [9]. Thus, when they consume carbon, they had already broken down these long bonds and converted them into simpler form that can be easily degraded [10].

The Materials that have been depolymerized and contain phenol can be further degraded by hydroxylation using a single or multicomponent hydroxylases (MPH). Ring cleaving enzymes like catechol-1,2-dioxygenase are used also to catalyze the ortho-cleavage route for catechol degradation [11]. Catechol is an important intermediary not only in the breakdown of aromatic compounds derived from plant-based materials, but also in the degradation of pollutants [12]. The current study aims to detect the prevalence of *Pseudomonas* sp. in three potentially polluted spots and the enzymes responsible for phenol degradation capability in bacteria and the genetic variation of the catabolic genes related to the *phe* operon among the positive isolates.

2. MATERIALS AND METHODS

2.1 Bacterial Isolation and Identification

One hundred twenty-five soil samples have been collected from different polluted sites at Baghdad city for the isolation of the most effective *Pseudomonas* sp. isolates in phenol-degradation. Eighty-nine (89) samples were collected from Midland refineries company (MRC)/ Daura refinery, twenty-one samples were collected from private electricity generators and fifteen samples were collected from different farm lands during the period from April 2021 to August 2021. Mineral salt medium (a liquid enriched medium) [1] and a number of differential and selective media like macConkey agar and *Pseudomonas* agar base media which were used to grow and diagnose the collected samples subsequently. VITEK system (BioMérieux, France) has been used for diagnosing samples by using the card VITEK® 2 GN ID Card.

The broth of the bacterial isolates was cultured overnight in nutrient broth medium (N. B), then they were subjected for DNA extraction, by using ABIO pure TM kit (Alliance Bio, USA). The concentration and purity of the extracted DNA were measured using Nanodrop. All the previously diagnosed isolates were subjected to DNA extraction and samples have been additionally diagnosed by PCR using the housekeeping gene 16S rDNA to confirm their identities [13] (Table 1). The reaction components included; 12.5 µl of GoTaq®Green Master Mix (Promega, USA), 1 µl of each sense and anti-sense primer (Macrogen), 2 µl of DNA templates of isolated bacteria, then the volume was completed to 25 µl by adding 8.5 µl of nuclease free water. PCR reaction was then carried out using the following program: 2 minutes of initial denaturation at 95 °C, followed by 25 cycles of three stages: denaturation at 94 °C for 20 seconds, annealing at 58 °C for 20 seconds, and extension at 72 °C for

Table 1. Primer sequences used in the current study

Gene name	Primer Name	Sequence 5′→3′	Product Size	Reference
16s rDNA	F	AGAGTTTGATCCTGGCTCAG	956 bp	[13]
	R	CTTGTGCGGGCCCCCGTCAATTC		
***cat*1**	F	AAACCCGCGCTTCAAGCAGA	650 bp	Present study designated
	R	AAGTGGATCTGCGTGGTCAGG		
***cat*2**	F	TGATCGAGATGGACCGTGAC	821 bp	Present study designated
	R	TCAGGTCAGCACGGTCATGAA		

40 seconds. Finally, final extension temperature was adjusted to 72 °C for one minute. PCR results were visualized on gel electrophoresis of 1 % agarose and showed sharp single bands on the gel. PCR products for the amplified gene (stored at -20 ºC) was sequenced by sending 20 µl of the amplified product to Macrogen, Korea. Data were analyzed using Geneious software and the results were read by comparing them with the NCBI control standard strains. Query, pairwise alignment and identity, were anatomized with same software.

As for phenol treatment, the positive isolates for HKG have been tested for phenol degradation capability by adding different concentrations of phenol (100 ppm to 1500 ppm) to the mineral salt medium to examine their tolerance to phenol.

2.2 Detection of Catechol Dioxygenases using Conventional PCR

DNA templates of the targeted isolates were used to amplify the catechol dioxygenase enzymes using specific primers: catechol 1,2 dioxygenase (*cat*1), and catechol 2,3 dioxygenase (*cat*2) genes by conventional PCR. After many optimization trials, the two set primers were subjected to the following conditions: Initial denaturation at 94 °C for 3 minutes, followed by 35 cycles of three stages, including denaturation at 94 °C for 30 seconds, annealing at (56 °C to 60.2 °C) for one minute, extension at 72 °C for two minutes. Then final extension was set for 7 minutes at 72 °C.

The primer sequences that used are summarized in (Table 1). After sending those PCR products to Macrogen, Korea. Data were analyzed using Geneious software and the results were read by comparing them with the NCBI control standard strains. Query, pairwise alignment and identity, were anatomized with same software.

3. RESULTS AND DISCUSSION

3.1 Bacterial Isolation and Identification

After the collection of samples, they have been classified according to the source of collection (89 samples from Daura refinery, 21 samples from the soils of private electricity generators, and 15 samples from farm lands at Baghdad city, these sites are mostly exposed to pollution due to factories in that area with the farm land as least polluted site (Table 2).

The diagnosis of soil samples using enriched and selective media and VITEK system revealed that 29/89 (32.5 %) of Daura refinery were *Pseudomonas aeroginosa* and only one sample was *Pseudomonas putida* 1/89 (1.1 %). on the other hand, none of the samples from generators were *P. aeroginosa* (0 %) and 5/21 (23.8 %) samples were *P. putida*; while 5/15 (33.4 %) samples of farm lands were *P. aeroginosa* 5/15 (3.33 %) and no sample were detected for *P. putida* (0 %). That means that this step detected forty (32 %) phenol-degrading isolates from the total number of isolates (Table 2).

Phenol degradation capability of the targeted isolates have been tested and the results revealed that all the forty isolates that previously diagnosed as *Pseudomonas* sp. (100 %) were able to degrade phenol till the concentration of 600 ppm.

Only four isolates (10 %) of the total number were characterized to tolerate phenol to a concentration exceeded 1200 ppm and only one isolate (2.5 %) tolerated phenol to the maximum concentration (1500 ppm).

A housekeeping gene 16S rDNA was used for genotypic identification of *Pseudomonas* spp. isolates and all the isolates (100 %) showed a

Table 2. Distribution of *Pseudomonas* species according to the collection source

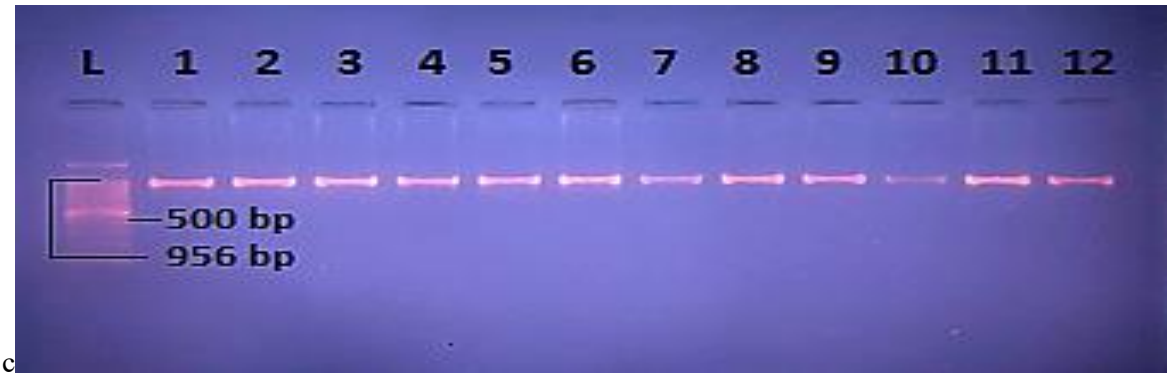
Source of sample	Total no. Pre-Diagnosis	*Pseudomonas aeruginosa*	*Pseudomonas putida*	Total number
Daura refinery	89	29 (32.5 %)	1 (1.1 %)	30 (2.37 %)
Private Generators	21	0 (0 %)	5 (23.8 %)	5 (23.8 %)
Farm lands (not polluted)	15	5 (3.33 %)	0 (0 %)	5 (3.33 %)
Total number	125	34 (27.2 %)	6 (4.8 %)	40 (32 %)

positive result. Clear bands were shown on agarose gel and expected size was 956 bp (Figure 1A) and they were matched with 100 bp DNA ladder. The Blast hit is presented in figure (1B) clarifies the amplified size from the part of the gene size. The pairwise identity was 99.89 %, which represents the residue percentages that were identical to gaps versus non-gap residue. Some of the differences appeared between local isolate and recorded NCBI strain as compared with WHO stander strain MH114980 (Figure 1C).

3.2 Detection of Catechol Dioxygenases Using Conventional PCR

The results of detecting *cat*1 gene showed that all

A.



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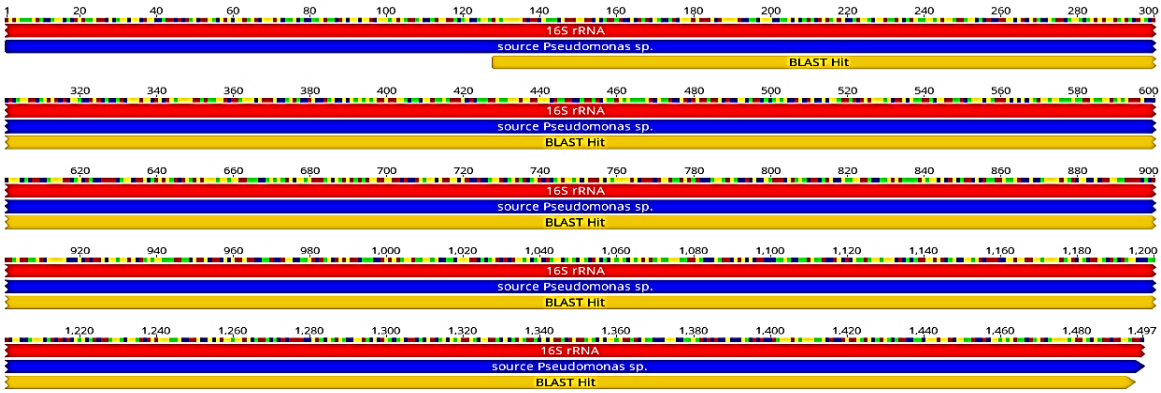
B.



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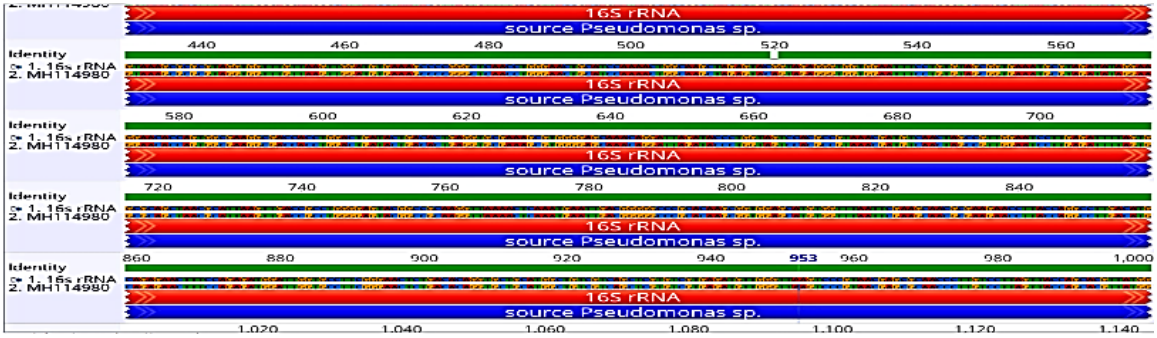


Fig 1. A. Agarose gel electrophoresis (1 % percent agarose, 5 V/cm for 90 minutes) for 16S rDNA gene (amplified size of 956 bp) vs. DNA ladder B. 16S *Pseudomonas* spp: precise molecular size 1368 bp, Blast hit: interval: 128 ->1495 from the original 16s DNA related to the NCBI standard strain MH114980 C. Pairwise identity 99.89 % as compared with WHO stander strain MH114980.

the 40 (100 %) isolates were positive for this gene (Table 3). Clear bands appeared in agarose gel and expected size was 650 bp as in (Figure 2A). The blast hit is presented in (Figure 2B) which clarifies the amplified size from the part of the gene size. The sequences of *cat*1 gene for *P. putida* local isolate was displayed in (Figure 2C). Sequence comparison was done between DNA segment of the current study and the standard strain CP016212 in which pairwise identity reached the percentage of residues that were similar in alignments with gap vs. no gap residues was 98 % percent. There were just a few discrepancies between the local isolates and the reference strain.

The results of *cat*2 gene amplification showed that this gene was located in six isolates (15 %) of the total number as shown in (Table 3) and Figure (3A). The result was represented with sharp single bands with expected size of (821bp). Blast hit is presented in figure (3B) which illustrated the precise amplified region from the complete gene. The resulted sequence has aligned with a sequence from NCBI database under the reference ID APO15030, and the results of sequence comparison represented in Figure (3C).

This study was conducted for the isolation of *Pseudomonas* sp. from the soils contaminated with phenolic compounds at Baghdad city and the evaluation of their capability to degrade phenol. Polluted soil samples were collected from different regions at Baghdad city, Iraq. These samples were cultured on the mineral salt medium [1] with the presence of phenol in this enriched medium for the isolation of phenol degrading bacteria. Further culturing was done using differential and selective media for the diagnosis of these isolates, beside diagnosing them by the VITEK system and the HKG using 16S rDNA gene at the molecular level. Soil indigenous microbial communities have a flexible capability in biodegrading hydrocarbonic compounds in oil-polluted soils as previously demonstrated [1,14]. The results of this study showed higher frequency of *P. aeruginosa* among the isolates harvested from Daura refinery. This results agreed with a previous study done by Khatoon and Malik [15].

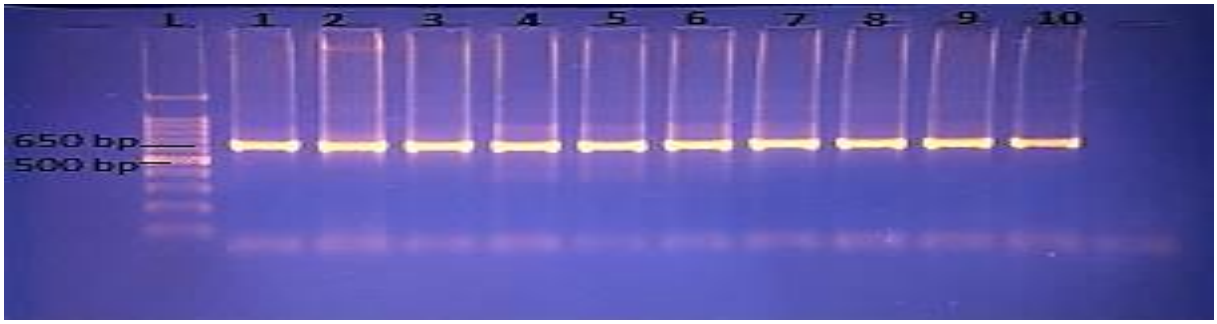
Many researchers have investigated the degradation ability of phenol by various *Pseudomonas* species under aerobic and anaerobic conditions. A previous study suggested that the isolated *Pseudomona*s degrades phenol aerobically via the ortho-cleavage route [16]. The degradation process begins with the creation of catechol, which occurs when phenol hydroxylase (monooxygenases) attaches a hydroxyl group to the benzene ring in the ortho position. In addition to monooxygenases, dioxygenases which are classified into two families, intradiol and extradiol dioxygenases can degrade catechol rapidly by catalyzing the oxidative cleavage of catechol by any pathway resulting in the rapid elimination of the intermediate metabolites [17]. The primary intermediate generated during the biodegradation of phenol by various microbial strains is catechol. Catechol 1,2-dioxygenases catalyze the ortho-cleavage pathway [18] while catechol 2,3-dioxygenases catalyze the meta-cleavage pathway [19]. Both *P. aeruginosa* and *P. putida* isolates were found in this investigation and they were efficient as phenol degrading isolates because they have the enzymes responsible for this ability and also all of them were positive for the catechol 1,2 dioxygenase gene (*cat*1) targeted in the current study.

The catechol dioxygenases serve as part of nature's strategy for degrading aromatic molecules in the environment. They are found in the soil bacteria and involved in the transformation of aromatic precursors into aliphatic products. The

Table 3. Detection of Catechol Dioxygenases in *Pseudomonas sp*. isolates

***Pseudomonas sp*. isolates**	**Catechol Dioxygenase genes**	
	***cat*1**	***cat*2**
Pseudomonas aeruginosa isolates	34 (85 %)	4 (10 %)
Pseudomonas putida isolates	6 (15 %)	2 (5 %)
Total	40 (100 %)	6 (15 %)

A.



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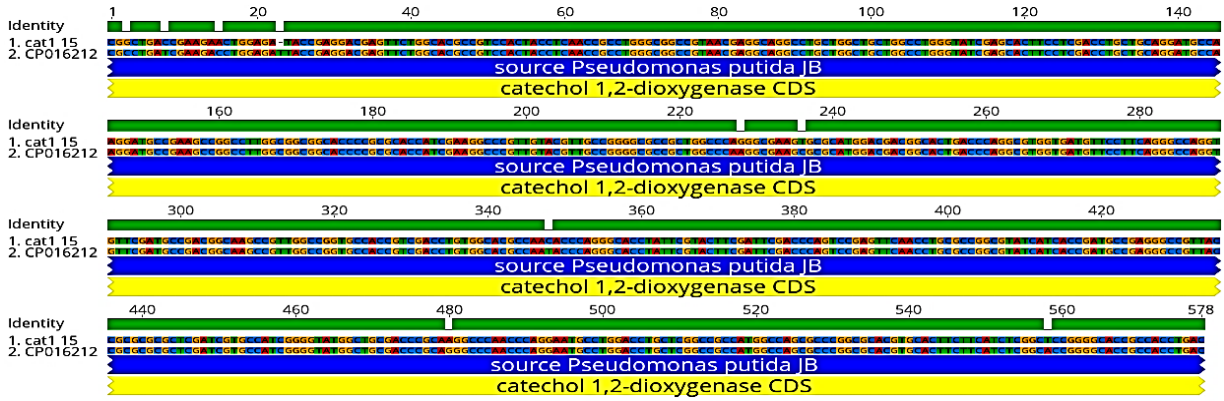

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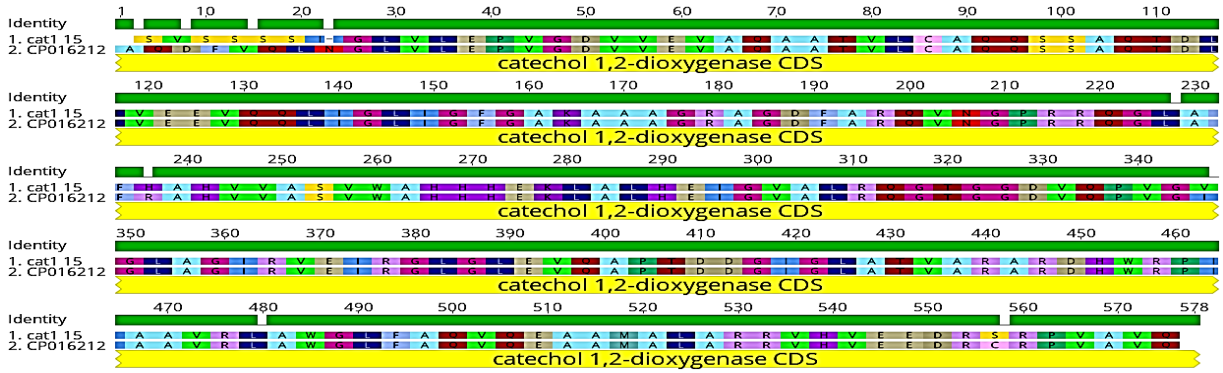


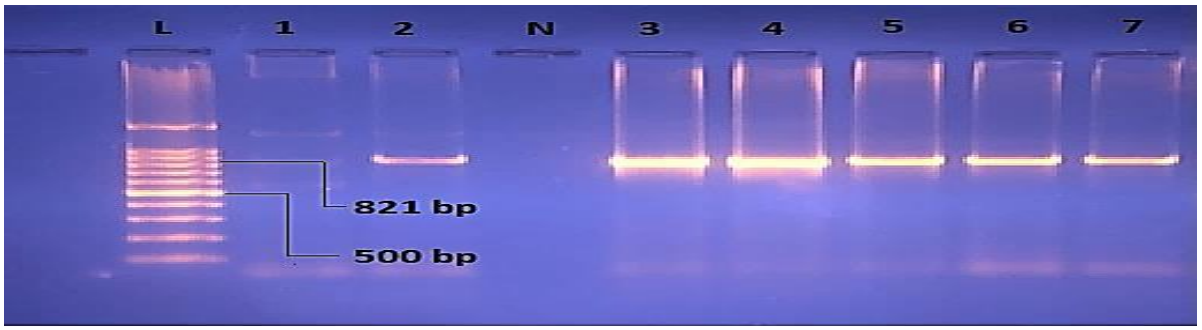
Fig 2. A. Agarose gel electrophoresis (1% percent agarose, 5 V/cm for 90 minutes) for *cat*1 gene (amplified size of 650 bp) vs. DNA ladder lane B: In the current investigation, a blast hit of the amplified gene. C: Pairwise identification CP016212 and DNA sequencing for the *cat*1 gene shows that the local isolate has few gaps.

intradiol cleaving enzymes utilize Fe (III), while the extradiol cleaving enzymes utilize Fe (II) and Mn (II) in few cases [20]. In *Pseudomonads*, many of its induced enzymes are nonspecific and its metabolic pathway contains a high degree of convergence. This convergence of catabolic pathways allows them efficiently utilizing a wide range of growth substrates, while the non-specificity of these induced enzymes allows for the simultaneous utilization of many similar substrates without an extra redundant genetic coding for enzyme induction [21].

4. CONCLUSION

In the present study, two species of *Pseudomonas,*

A.



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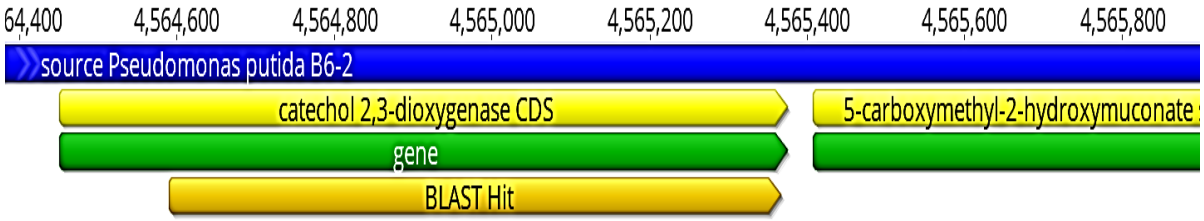

C.

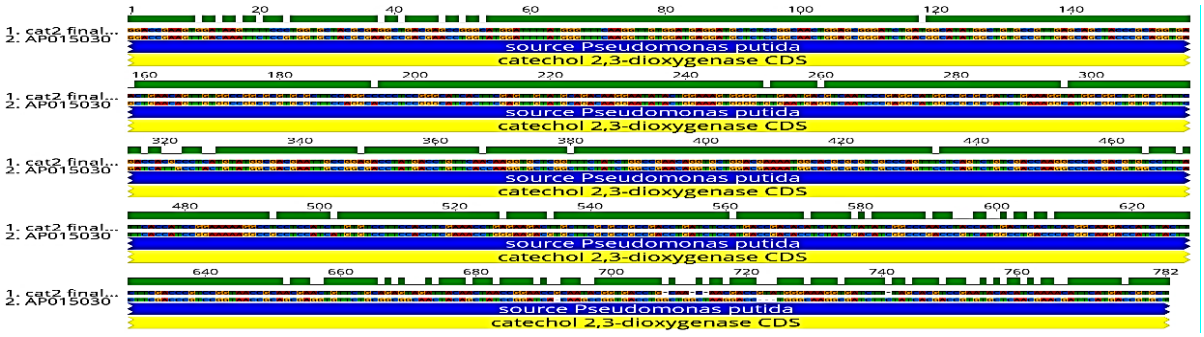

Fig 3. A. Agarose gel electrophoresis (1 percent agarose, 5 V/cm for 90 minutes) of the *cat*2 gene (amplified size of 821 bp) vs. DNA ladder lane. B: The magnified Blast Hit gene in the current study. C: Pairwise identity APO15030 and *cat*2 gene DNA sequencing, which revealed a few gaps in the local isolate.

P. aeruginosa and *P. putida* were isolated from soils contaminated with phenolic compounds. Phenol degradation capability of the targeted isolates have been tested and vast majority of them were effectively capable of degrading phenol to the concentration of 1200 ppm and only one isolate tolerated phenol to the maximum concentration (1500 ppm) which is the isolate number 15 which is considered the most effective isolate in phenol degradation in or study. The previous findings can be seen as an important tool in the treatment of soils and water that polluted with phenol. The existence of such enzymes in the tested isolates reflects their catalytic potential in degrading such pollutants.

5. ACKNOWLEDGMENTS

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6. CONFLICT OF INTEREST

The authors declared no conflict at interest.

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