EXPRESSION OF DMPN IN *PSEUDOMONAS PUTIDA* FOR DEGRADATION OF PHENOL IN SOIL

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ABSTRACT

Background: *Pseudomonas putida* can efficiently degrade phenol. The aim of this study was to determine the level of expression of the decomposition of the biological bacteria pseudo-foul pseudomonas putida affected by pesticides. Methods: the study included the collection of 125 different soil samples from the soil surrounding the roots of different plants. Culturing and biochemical tests, also diagnostic devices using the Vitek system for diagnosis of *Pseudomonas putida*. genetic variation of genes responsible for biodegradation, polymerase chain reaction (PCR) was used to detect *DmpN* gene for molecular diagnosis and RT-PCR for gene expression. the results of the PCR showed that *DmpN* gene (199bp) exists in all *P. putida.*In conclusion: the study showed the importance of *Pseudomonas putida* for biological decomposition of phenol from the soil surrounding the roots of the plants.

Keywords: decomposition, bioloyicoal bacteria, pesticides, soil samples, genes, PCR.
Part of M. Sc. Thesis of the 1 st authar.

تعبير لل DMPN في *PSEUDOMONAS PUTIDA* لأ نحلال الفينول في التربة

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المستخلص

بكتريا *Pseudomonas putida* تستطيع ان تحلل مركبات الفينول بكفاءه عالية. اجريت هذه الدراسه وذلك لتحديد مستوى تعبير الجينات المحلله لمركبات الفينول لبكتريا pseudomonas putida التي تتكون بواسطة المواد الكيميائيه في التربه. تضمنت هذه الدراسة جمع عينات تقدر ب 125 عينه من ترب مختلفه وتكون محيطه بجذور لنباتات مختلفه. زرع العينات والفحوص الكيميائيه وكذلك استعمال نظام الفايتك لتشخيص *Pseudomonas putida*. الاختلافات الجينيه المسؤوله عن تحلل مركبات الفينول تم تحليلها بواسطه تفاعل سلسلة البلمرة. وكذلك التعبير الجيني لجين *dmpN* وجين *phe* بواسطة النسخ الرجعي. اظهرت هذه الدراسة ان جين *dmpN* و *phe* يوجد في جميع العزلات البكتريا وبينت عذه الدراسة اهمية بكتريا *Pseudomonas putida* في عملية التحلل البايولوجي لمركبات الفينول في التربه المحيطه بجذور النباتلت.

كلمات مفتاحية: فحوصات كيميائية، النسيج الرجعي، سلسلة البلمرة.

جزء من رسالة ماجستير للباحث الاول.

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INRODUCTION

Since large amounts of phenolic compounds are produced naturally, phenol is expected not to show recalcitrant behavior in the environment. More over rapid disappearance of phenol added to environment samples has been observed, although phenol degradation in natural environment is not well documented (29). The aerobic degradation of phenol has been studied intensively. Many bacteria, fungi and yeasts that are capable of metabolic degradation of phenol or that can use it as sole source of carbon and energy have been isolated (4). The bacterial degradation of phenol under aerobic conditions starts with formation of catechol. The pathway of phenol degradation has also been studied in *Pseudomonas sp*. CF600, which also uses metacleavage. The genes of phenol degradation have been analyzed (3). The metabolic pathway of the phenol-degrader *Pseudomonas putida* BH by introducing the catechol 2,3 oxygenase encoding gene (phe B), isolated from the chromosome of P. putida BH, as a recombinant plasmid (Pbh500), with waste water that contained low concentration of xenobiotic compound, P. putida BH (Pbh500) could be used to accelerate phenol degradation (25). *Pseudomonas putida* strains have the catabolic potential to use many alternative carbon sources in the diverse and often inhospitable soil, rhizosphere and aquatic systems in which they thrive (8). This metabolic versatility is frequently expanded through possession of plasmids or other mobile DNA elements that encode auxiliary pathways for the catabolism of toxic aromatic compounds. However, not all carbon sources are metabolically equivalent. Sophisticated global carbon repression control (CRC) networks have evolved to ensure that when presented with a mixture of substrates, those that provide the highest metabolic return are preferentially and hierarchically consumed (7, 13 , 26). The dimethylphenol *dmp*-system for the catabolism of phenol and (methyl)phenols has been extensively used to probe specific and global regulatory networks that impact assimilation of phenolic aromatic compounds (27). The *dmp*-system is carried on the pVI150 IncP-2 mega-plasmid of *P. putida* CF600 and is composed of the *dmpR* gene for the master regulator of the system and the Dmp enzymes encoded within the closely linked but divergently transcribed *dmp*-operon. The *dmp*-operon encodes the entire suite of specialized enzymes for the dissimilation of (methyl)phenols to central metabolites. The first six genes (*dmpKLMNOP*) are required for production of a multicomponent phenol hydroxylase for conversion of (methyl)phenols to catechol or methyl-catechol, which are then sequentially processed to pyruvate and acetyl-CoA via the enzymes of a *meta*-cleavage pathway encoded by the *dmpQBCDEFGHI* genes (22,28)

MATERIALS AND METHODS

One hundred twenty five soil samples were collected from the rhizosphere of plants growing at different sites of Iraqi soil district in Pomegranate and olive Orchards Near the root (South Baghdad Al- Yusufiyah) during the period from October 2016, till the of June 2017. Fifteen bacterial isolates were obtained from soil samples; the 40 isolates with the highest capacity for hydrocarbons biodegradation phenol were selected. They were cultured on nutrient agar and MacConkey media and tested by gram stain and then streaking on Modified mineral salt medium with agar (MMSM-H) that colony on this media considered as *Pseudomans putida.* The isolates were cultured on Cetrimide agar as a selective medium for *P.putida* ,along with the other media namely ,Kings A and Kings B agar. Identification of *P. putida* isolates was done by a number of biochemical and cultural tests. *P. putida* was obtained to be motile, producing pyocianin (a water- soluble phenazing pigment on king A medium).Growth at 41°C and no growth was found at 4°C, oxidation of maltose and mannitol, nitrate production, ornithine and arginine decarboxylation, indolphenol oxidation reaction and liquefaction of gelatin. The VITEK 2 is an automated microbiology system utilizing growth-based technology. It was using the identification card for Gram negative strains (ID-GNB).

Optimization conditions for biodegrading

Degradation of phenol was studied on a phenol supplemented minimal salts medium (MSM) containing: KH_2PO_4, 2.25; K_2HPO_4, 2.25; $(NH4)_2SO_4$, 1.0; $MgC_{l2}.6H_2O$, 0.2; NaCl, 4;

FeCl3.6H2O, 0.02; CaCl2, 0.01 and phenol 0.1, in g/L; pH= 7. P. fluorescens and P. putida were inoculated in a 250-ml flask containing 100 ml of MSM separately. The experimental studies were conducted in shake flasks with agitation at a rate of 150 rpm. Bacterial growth was determined in terms of cell mass by measuring optical density at a wavelength of 600 nm using a UV-1800 UV/VIS Spectrophotometer. The phenol concentrations in medium were determined by the UV spectrophotometer at a wavelength of 272 nm after incubation period.

RESULTES AND DISCCUATION

Molecular identification

Genomic DNA extraction: Genomic DNA was extracted from *P. puptida* isolates by using genome DNA purification kit (Disbio / China). Extraction genomic DNA from isolates that was confirmed as bands by gel electrophoresis on 0.8% agarose. DNA concentration and purity were measured by Nanodrop spectrophotometer, all the isolates had DNA concentration between (70-150 ng/µl) Reverse transcription quantitative PCR (RT-qPCR) is distinguished from other methods for gene expression because of accuracy, sensitivity and fast results. This technology has established itself as the golden standard for gene expression analysis. It is important to realize that in a relative quantification study, the experiments are usually consequential in comparing the expression level of a particular gene among different samples . (RT-qPCR) applied in the present experiment utilizes the SYBR green; a fluorescent dye which recognizes any double stranded DNA including cDNA. The amplification was recorded as Ct value (cycle threshold). In terms of gene expression, high Ct values indicate low gene expression and low Ct value indicates a high gene expression. The chief purpose of this step was to measure the gene expression of the *DmpN* genes and compare the gene expression in different environmental factors in order to improve the changeover conditions growth of bacteria *P.putida* leads to change over of gene expression. The extracted DNA of isolate also used in the PCR reaction with *DmpN* gene primers to prove the presence of this gene in the other species of *P. putida.*

Total RNA Extraction

The experiment of quantitative PCR reaction was done by using 4 isolates of *P. puptida* which gave highest absorbability for dimethylphenol producing. Total RNA was successfully extracted from all samples. The concentration of total RNA ranged from 80 to 120 ng/ µl ,and the purity of total RNA samples ranged from (1.6 to1.9) A good yield with a high concentration of total RNA depends on the extraction conditions whereby strict aseptic techniques must be used. The utilization of Trizol in the Total RNA extraction from bacteria is well recommended.

DNA reverse transcription

A common primer reaction was applied since it was needed to have cDNA for the gene in the study. The efficiency of cDNA concentration was assessed through the efficiency of qPCR conducted later on All steps were associated with perfect yield reflecting efficient reverse transcription.

Real time PCR quantification of *DmpN* Expression

quantitative RT- PCR assay analyzed the mRNA expression of *DmpN* genes by comparing the bacterial growth with different environmental factors by using the Ct values of genes amplification were recorded from the software of quantitative RT PCR. The calculation of gene expression fold change was made using relative quantification.This depends on normalization of Ct values calculating the ΔCt which is the difference between the mean Ct values of replica of *DmpN* cDNA.

Results

Table 1.Distribution of *P. putida* isolates in soil samples

Sample type	No. of Sample	No. of *P putida* on MSMM media
Soil sample	125	50

Table 1 shows the distribution of *P. putida* isolates in soil samples which show from 125 samples the no. of p. putida that isolated in MSMM media was 50 samples.

Figure 1. show P. putida on king B agar

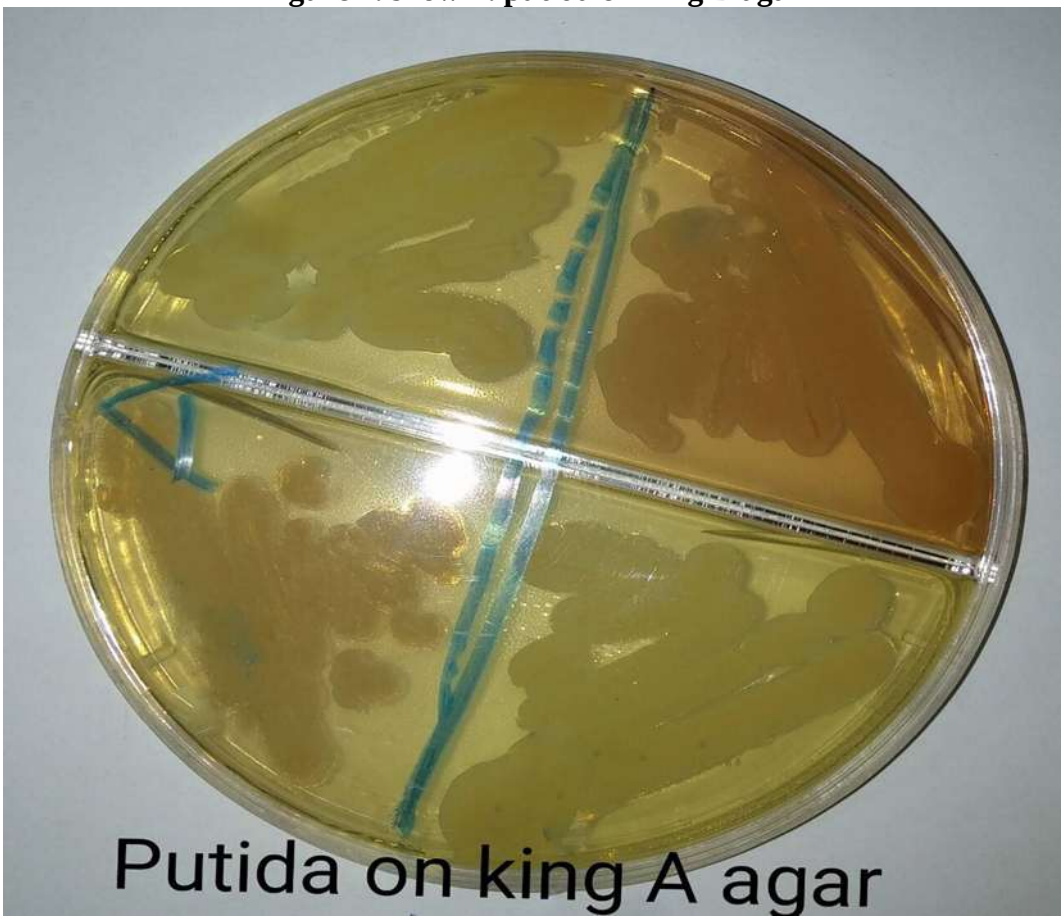

Figure 2. show P. putida on king A agar

Table 2. Distribution of *P. putida* isolates in MSMM media and Vitek2 test

Sample type	No. of Sample	No. of *P putida* in MSMM	No. of *P putida* in Vitek2
Soil sample	125	50(40%)	42(33.6%)

Table 2 shows the distribution of P. putida which isolated from the soli and isolated then in MSMM media then more accurate diagnosis of P. putida by biochemical test (vitek2) system which show that the no. of P. putida by vitek2 system was 42 (33.6%).

Table 3. Effect of different incubation periods, Ph, Temperature and concertation on the biodegradation of phenol

concertation	Control	St.day	2nd day	3rd.day	4th.day	5th.day	6th.day	7th.day	8th.day
3200	0.018	0.03	0.035	o.o4	0.043	0.047	0.049	0.06	0.05
4000	0.018	0.036	0.039	0.043	0.045	0.05	0.053	0.055	0.057
4800	0.018	0.04	0.043	0.045	0.048	0.051	0.055	0.058	0.061
5600	0.018	0.047	0.05	0.054	0.058	0.063	0.069	0.078	0.078

Table 3 shows the effect of different incubation periods, PH, temperature and concentration on the biodegradation of phenol which show that the biodegradation of phenol by P. putida increases by increases concertation and incubation period, also by increasing optical density.

Table 4. Biochemical test results for *P.putida*

Biochemical test	Result
Motility	+
Oxidase production	+
Catalase production	+
Lactose fermentation	-
Gelatin Liquefaction	-

Table 4 shows the biochemical test for P. putida which show that positive motility, oxidase production positive, also catalase positive. In the other hand lactose fermentation and gelatin liquefaction was negative.

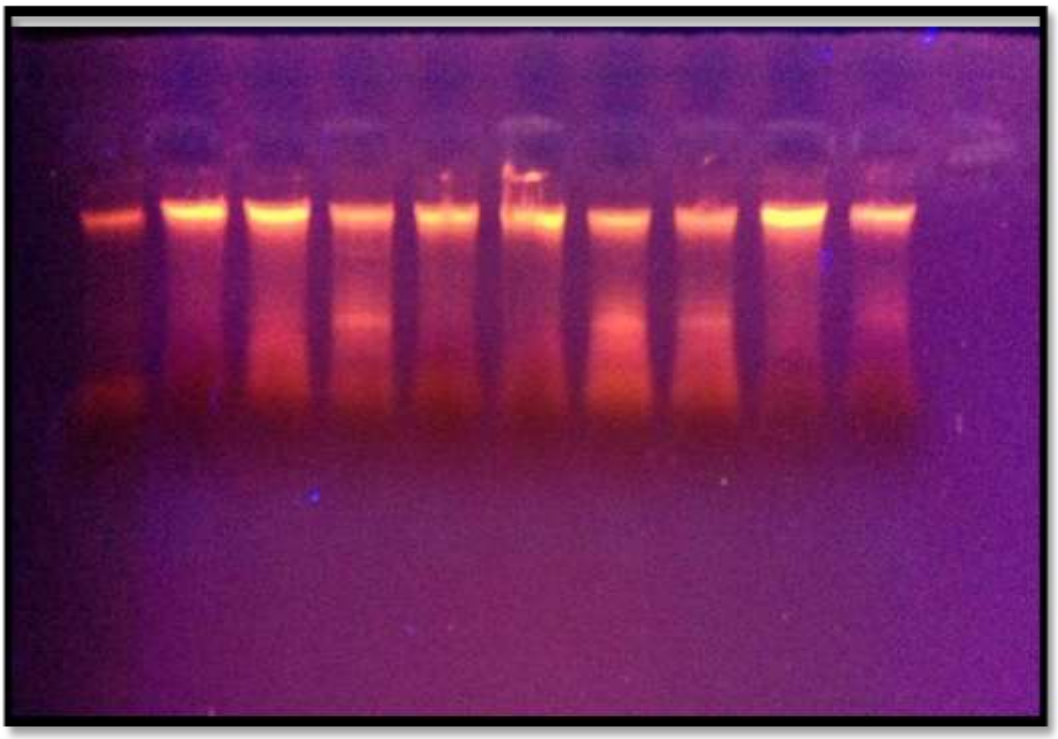

Figure 3. Agarose gel electrophoresis of extracted DNA to check purity and integrity. Lane 1-10: DNA of different *P.putida* isolates, Lane C: Negative control. (70 V/ 30 min.)

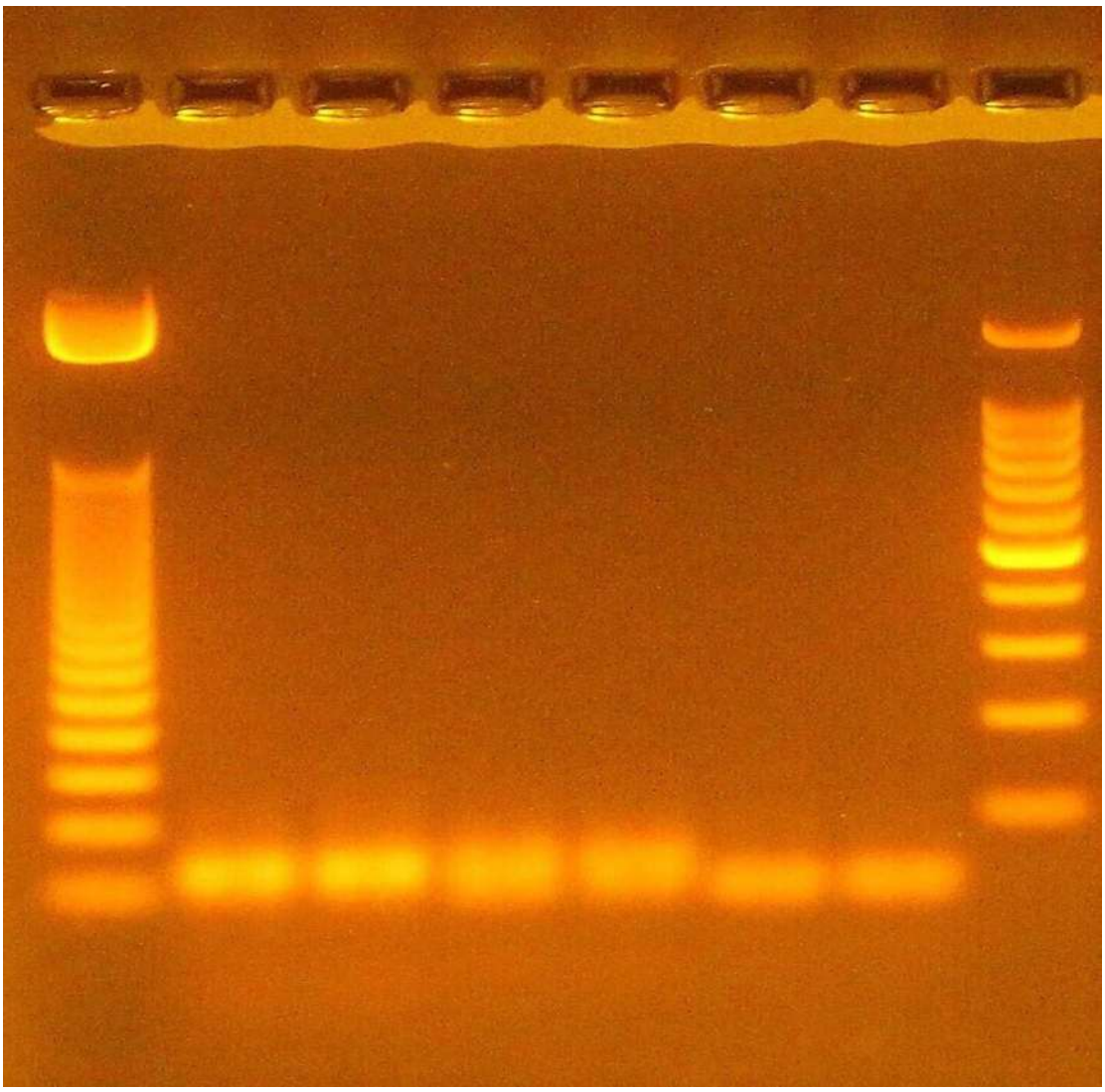

Figure 4. Agarose gel electrophoresis of PCR amplification products of *P. putida DmpN* 199. Lanes 2-7 : positive results, lanes: 1,8 ladder 1500 bp

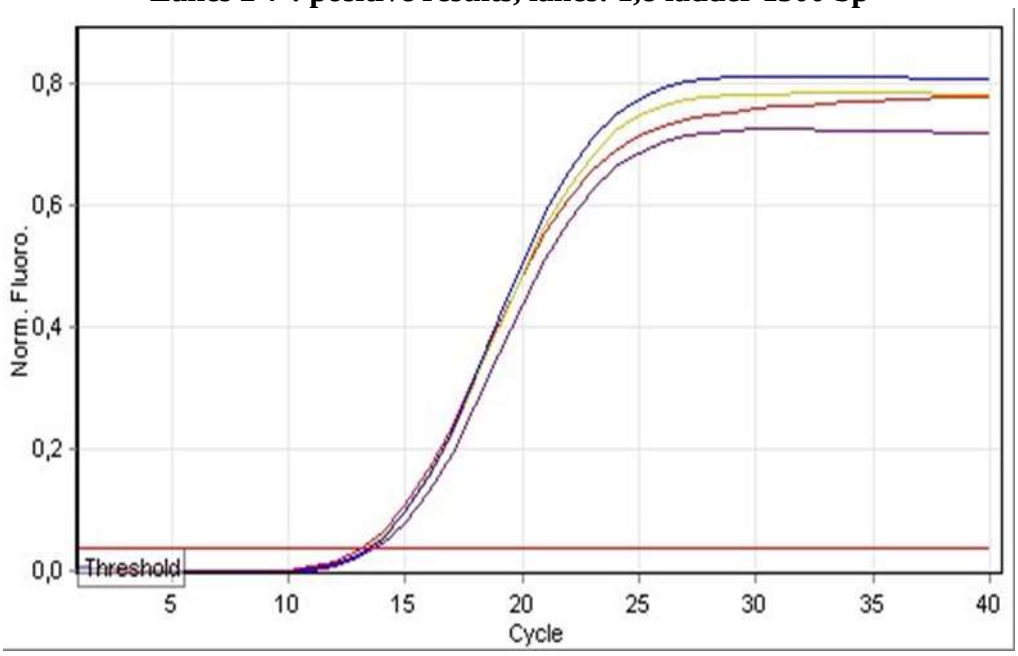

Figure 5 *DmpN* gene amplification plots by qPCR Samples included 48hr factor .Ct values ranged from 13.10 to 13.75. The photograph was taken directly from Rotor-Gene qPCR machine .

Table 5. Fold of *DmpN* expression Depending on $2^{-\Delta Ct}$ Method for Incubation period factor according concentration

Groups	concentration	Means Ct of pheU2	Means Ct of DCT	2-ΔCt	Fold of gene expression
Zero time	**O**	**9.27**	**-18.71**	**0**	**1.0**
	2400	**9.23**	**-19.39**	**-0.67**	**1.6**
	3200	**9.19**	**-19.00**	**-0.28**	**1.2**
	4000	**9.24**	**-18.88**	**-017**	**1.1**
3 Days	**O**	**9.14**	**-19.44**	**0**	**1.0**
	2400	**9.12**	**-19.59**	**-0.14**	**1.1**
	3200	**9.18**	**-19.22**	**0.21**	**0.9**
	4000	**9.17**	**-19.65**	**0.20**	**1.2**
8Days	**O**	**9.40**	**-19.18**	**0**	**1.0**
	2400	**9.31**	**-19.28**	**-0.10**	**1.1**
	3200	**9.39**	**-19.76**	**0.41**	**0.7**
	4000	**9.15**	**-19.16**	**0.01**	**1.0**

Table 4, 5, 6 shows the expression DmpN gene according to incubation period and concentration which show In incubation period factor The mean Ct value of *DmpN* cDNA amplification was **(9.27)** in the control. The Ct values in 3 days hours was a mean **(9.19)**. While Ct values in 3 days was mean **((9.24)**, there was a significant difference in the mean Ct values between the different incubation period factor. Expression of the *DmpN* gene was not equal in both 8days The fold of gene expression of *DmpN* gene for the four isolates in 48hr mean value(**9,19**), this result was higher than the fold of *DmpN* gene in 8 days with mean value (**9.40**). This is important in reflecting the original mRNAs present in the samples. It is evident from these results that the incubation period factor is associated with the highest copy number of mRNAs reflecting its higher expression. and it is important evidence that *DmpN* gene expression increases in 48hr. Microbial metabolism is an important factor in determining the magnitude and duration of decomposition and concentrations of phenolic allelochemicals in soils. In this study, we isolated microbes that can effectively decompose phenolics from soils Our results reveal that soil conditions are important factors for the growth and metabolism of microbes, as well as for microbial decomposition of soil phenolics. The growth and functions of the isolates from soils are largely dependent on medium conditions of pHs, temperatures and metal ions.In a study show that the two strains of *Ps. putida* 4CD1 and 4CD3 could not grow at pH 4Æ0 and 3Æ0 conditions, which are common in most acidified soils in our area; while all three strains of *Pseudomonas* could not grow in soils contaminated with Co2+ (31). Degradation of toxic contaminants by soil microbes has been studied extensively, such as degradation of 2,4-dichlorophenol (5), 2,4-dichlorophen-oxyacetic acid (21), 2,4,6-trichlorophenol (11), 2,4,6-trinitrotoluene (9), atrazine (24),benzoxazolinone and benzoxazinone (10), dichloro-diphenyl-richloroethane (DDT, 1), naphthalene (32), phenan-threne (31), polyhydroxyalkanoates (17) and other compounds (14,30 , 19).The three bacterial species identified as *Ps. putida*,*Ps. nitroreducens* and *R. glutinis* are common microbes inthe environments and have been demonstrated to be effective indecomposing and transforming toxic organic compounds (,16, 6, 15, 12, 18 , 20 , 23). Another study show Biodegradation of phenol with pure culture of *Pseudomonas putida* was investigated. P. putida (PTCC 1694) was grown in facultative anaerobic condition at 27 degrees C and media pH value of 7. The effect of initial phenol concentration on the biodegradation rate was studied. The initial concentrations of phenol varied from 300 to 1000 mg/l. Experiments were performed for the duration of seven days while daily samples were withdrawn. The initial rate of biodegradation of phenol increased with initial concentration of 300-500 mg/l. Further increase in phenol concentration resulted in a slight decrease in the rate of biodegradation due to phenol inhibition. It was observed that by increasing the concentration of phenol, the lag phase was prolonged. Phenol is known to be an inhibitory substrate, thus Monod, Haldane and logistic kinetic models were applied to evaluate the growth kinetic parameters. The Monod model was unable to

present the growth parameters over the defined concentration range. However, Haldane and logistic models perfectly fitted with the experimental data. The yield coefficients for the growth on phenol at concentrations of 300, 500, 700 and 1000 mg/l were 0.177, 0.062, 0.035 and 0.012 mg/mg, respectively (2). In a study Participation of *Pseudomonas putida*-derived methyl phenol (dmp) operon and DmpR protein in the biodegradation of phenol or other harmful, organic, toxic pollutants was investigated at a molecular level. Documentation documents that P. putida has DmpR protein which positively regulates dmp operon in the presence of inducers; like phenols. From the operon, phenol hydroxylase encoded by dmpN gene, participates in degrading phenols after dmp operon is expressed. For the purpose, the 3-D models of the four domains from DmpR protein and of the DNA sequences from the two Upstream Activation Sequences (UAS) present at the promoter region of the operon were demonstrated using discrete molecular modeling techniques. The best modeled structures satisfying their stereo-chemical properties were selected in each of the cases. To stabilize the individual structures, energy optimization was performed. In the presence of inducers, probable interactions among domains and then the two independent DNA structures with the fourth domain were perused by manifold molecular docking simulations. The complex structures were made to be stable by minimizing their overall energy. Responsible amino acid residues, nucleotide bases and binding patterns for the biodegradation, were examined. In the presence of the inducers, the biodegradation process is initiated by the interaction of phe50 from the first protein domain with the inducers. Only after the interaction of the last domain with the DNA sequences individually, the operon is expressed. This novel residue level study is paramount for initiating transcription in the operon; thereby leading to expression of phenol hydroxylase followed by phenol biodegradation. The dmp-system encoded on the IncP-2 pVI150 plasmid of Pseudomonas putida CF600 confers the ability to assimilate (methyl)phenols. Regulation of the dmp-genes is subject to sophisticated control, which includes global regulatory input to subvert expression of the pathway in the presence of preferred carbon sources. Previously we have shown that in P. putida, translational inhibition exerted by the carbon repression control protein Crc operates hand-in-hand with the RNA chaperon protein Hfq to reduce translation of the DmpR regulator of the Dmp-pathway. Here, we show that Crc and Hfq co-target four additional sites to form riboprotein complexes within the proximity of the translational initiation sites of genes encoding the first two steps of the Dmp-pathway to mediate two-layered control in the face of selection of preferred substrates. Furthermore, we present evidence that Crc plays a hitherto unsuspected role in maintaining the pVI150 plasmid within a bacterial population, which has implications for (methyl)phenol degradation and a wide variety of other physiological processes encoded by the IncP-2 group of Pseudomonas-specific mega-plasmids (21).

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