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Biodegradation of Chlorpyrifos by Some Bacterial Strains and Screening Their Degraded Genes

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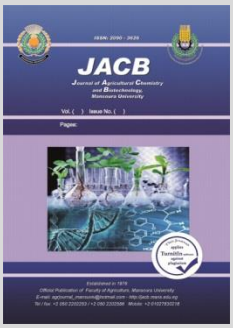

ABSTRACT

Biodegradation is a natural process, where the degradation of a xenobiotic chemical or pesticide by an organism is primarily a strategy for their survival. Pesticides are degraded in the environment principally by the action of microorganisms. Evaluate the ability of three bacterial strains to chlorpyrifos-degrade, determine the chlorpyrifos degrading genes in the isolates, study their biochemical mode of degradation. Three bacterial strains i.e., *Pseudomonas fluorescens, Rhizobium leguminosarum,* and *Bacillus megaterium* were detected in Luria-Bertani (LB) medium degrading Chlorpyrifos at day 14. Microbiological, biochemical studies and genetic factors in biodegradation were studied. *P. fluorescens* had the highest efficiency with 58.90%, then *R. leguminosarum* 56.72%, and finally *B. megaterium* with 50.69%. According to the genetic similarity for degradation genes, *B. megaterium* and *P. fluorescens* have nearly the same, while *R. leguminosarum* has another pathway. *P. florescence* and *R. Leguminosarum* strains were the highest in the degradation process which analyzed the Chlorpyrifos component to 3,5,6-trichloro-2-pyridinol (TCP) and Chlorpyrifos Oxon that has a little toxic effect on the ecosystem. With the obtained results, it is recommended to apply these strains on soil and plants to reduce the toxicity of chlorpyrifos in the environment.

Keywords: *Pseudomonas fluorescens; Rhizobium leguminosarum; Bacillus megaterium; OPD; MPD*

INTRODUCTION

Pesticides are produced all over the world to eliminate harmful organisms from plants and usually come into contact with soil. They remain in the environment for a very long time and accumulate into food chains decades after their application to soil (Aberdeen 1993). Among the various groups of pesticides that are being used, Organophosphorus pesticide chlorpyrifos has a broad range of insecticidal activity and is widely used in agricultural and domestic applications (Worthing and Hance 1991, and Racke 1993). It has been reported that chlorpyrifos (CPF) has high soil absorption coefficient than water solubility, and thus microbial degradation of CPF in these environments depends on the selection of appropriate bacterial species (Jayasri *et al*., 2014). To reduce the negative impacts of such toxic pollutants in the environment, it is desirable to degrade or at least quickly deactivate them in the environment especially by using naturally occurring bacterial microorganisms (Hindumathy and Gayathri 2013).

Chlorpyrifos (CPF) proved to be more readily biodegradable, where it is now used in preference to other pesticides. Biodegradation is a natural process, where the degradation of pesticides by an organism is primarily a strategy for their survival. Several Microbes hydrolyzed the organophosphorus effectively by cleave P–O in the phosphotriesters bond, P–S linkage in the phosphothiolesters, P–CN, or P–F of organophosphate pesticides (Yang *et al*., 2005). Various bacterial strains were isolated and reported for biodegradation of OPs *Serratia* sp. and *Pseudomonas aeruginosa* (Li *et al*., 2013 and Sasikala *et al.,* 2014). *Bacillus cereus, Bacillus subtilis, Brucella melitensis*, *Klebsiella* species, *Pseudomonas aeroginosa*, *Pseudomonas fluorescence*, and *Serratia marcescens* are capable of degrading 46-72% of chlorpyrifos as a sole carbon source in an aqueous medium after an incubation of 20 days (Lakshmi *et al.,* 2008).

Knowledge of the desired microbe's physiology, biochemistry, and genetics can help to improve the microbial process and achieve bioremediation with precision and little or no uncertainty or variability in microbe functioning. For many pesticides, a gene encoding an enzyme has been discovered, which will provide new inputs in understanding the microbial capability to degrade a pesticide and developing a super strain to achieve the desired biodegradation result in a short amount of time. Gene encoding for enzyme organophosphorus hydrolase (ophB, Opd) and methyl parathion hydrolase (Mpd) have been identified that can use OP compounds as a source of carbon, nitrogen, or phosphorus (Li *et al.,* 2007, Iyer *et al.,* 2013, Barman *et al.*, 2014 and Fayun *et al.,* 2017).

The objectives of the study were to evaluate the ability of *Pseudomonas fluorescens, Rhizobium leguminosarum, and Bacillus megaterium* strains to chlorpyrifos-degrade, determine the chlorpyrifos degrading genes in the isolates, study their biochemical mode of degradation and evaluate their CPF degradation patterns by HPLC.

MATERIALS AND METHODS

Strains used in this study had been previously isolated from the department of microbiology, Ain Shams University and from the department of soils, Alexandria

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University. Chloropyrifos® 48 % (0.0- diethyl 0-(35.6-Trichloro-2- Pyridyl) Phosphoro-thioate, OrganoPhosphorus group was purchased from DOW Agrosciences company, UK. Stock solutions of CPF (20 g.L) were prepared by dissolving CPF in acetonitrile and filtering through a membrane (0.22 mm).

Biodegradation of CPF:

Three types of bacterial strains *Pseudomonas fluorescens, Bacillus megaterium,* and *Rhizobium leguminosarum* were carried out in LB Broth medium containing a single concentrate of Chlorpyrifos pesticide and cultured in 250mL Erlenmeyer flasks on a rotary shaker (at 100 rpm) incubated at room temperature for about 14 days. The degree of biodegradation for each sample was measured through UV Spectrophotometer. Three ml of each sample were centrifuged immediately for 5 min at 10000 ×g. The concentration of pesticide (Chlorpyrifos 48%) was determined by measuring the absorbance at the maximum wavelength (290nm), using UV- visible light Spectrophotometry (Aggag *et al.*, 2018).

Screening degradation genes in bacterial strains:

Thirteen primer pairs were synthesized based on CPF degradation gene sequences published in the National Center for Biotechnology Information (NCBI https://www.ncbi.nlm.nih.gov/) for screening the possible CPF degradation genes as shown in Table (1).

The genomic DNA of the three strains was extracted with the Bacterial DNA kit (PureLink™ Microbiome DNA Purification Kit) and used as a template for the PCR procedure. PCR was carried out in a 25 µl volume and the amplification was performed in Primus 25 thermocycler. 95oC for 5 min; followed by 30 cycles of 95oC for 30-60 s, 52-60oC for 30-60 s, and 72oC for 60-90 s; and an extension at 72oC for 5-10 min. PCR products were migrated on 1% agarose gel electrophoresis, Data and cluster analysis were scored.

Table 1. Primers used in a polymerase chain reaction (PCR) to screen possible chlorpyrifos (CPF)-degrading genes.

Gene	Primers	Sequences (5'-3')
Opd(11)	OpdF(13)	ATGACATTTATCAATTCTGTAACAG
	OpdR(13)	TTACTTTCCTTCAAACCAG
	F196	CGCGGTCCTATCACAATCTC
	F450	CGCCACTTTCGATGCGAT
	R757	TCAGTATCATCGCTGTGACC
	R840	CTTCTAGACCAATCGCACTG
	R977	TCACTCTCAGTGGAATGAAGG
OphB(12)	OphBF(13)	TAATGGATCCGCCGCACCGCAGGT
	OphBR(13)	ATTAAAGCTTCTTGGGGTTGACGAC
	2350R	CGTCGTCGGCTGGGCAGGGT
	2351F	GCGTGCGGCCTACCTCGTTG
Mpd(10)	MpdF	GAATTCATATGCCCCTGAAGAAC
	MpdR	CGGAATTCTCACTTGGGGTTGAC

Detection of Chlorpyrifos and TCP by HPLC:

HPLC analysis was carried out for the two highly different species as well as mixed strains of degrading Chlorpyrifos pesticide. Chlorpyrifos and TCP were extracted from supernatant using an equal volume of dichloromethane (DCM) twice. Organic layers of DCM were aspirated, pooled, and evaporated at room temperature under nitrogen. The residues were dissolved in HPLC grade acetonitrile (1 mL) and then filtered through a flouropore TM filter membrane (0.45om 134 FH) to remove any particles. Extracted samples were analyzed on a Varian HPLC equipped with a ternary gradient pump, programmable variable-wavelength UV detector, column oven, and electric sample valve and ODS2 C18 reversed-phase column. Chlorpyrifos and TCP were detected at 290nm and 230nm wavelengths (Baskaran *et al.*, 1999).

RESULTS AND DISCUSSION

Results

Biodegradation of CPF:

The results of *Pseudomonas fluorescens* and *Rhizobium leguminosarum* revealed a high percent of degradation under different periods of the experiment. Degradation rates by *Pseudomonas fluorescens* were estimated after one hour, 6 hours, 1day, 7 days and 14 days. *Pseudomonas fluorescens* was higher than the other bacterial strains. However, the percent of degradation was lower than the other bacterial strains after 48, 72 hours. *Rhizobium leguminosarum* had a great potential of degrading Chlorpyrifos (CPF) pesticide. The results showed a high percent of biodegradation of *Rhizobium leguminosarum* bacteria. The percent of degradation was high after 2days, 3days, and 7 days. The percentage of degradation at 48 hours was 61.6198%. Whereas the percentage of the degradation at 72 hours was 60.9081% and the percentage of degradation at 7 days was 57.9812%. On the 7th day, the percent of degradation of *Pseudomonas fluorescens* and *Rhizobium leguminosarum* were nearly identical and were 58.2% and 57.9%. *Bacillus megaterium* had a good ability to degrade Chlorpyrifos pesticide but less than the other bacterial strains. The percentages of degradation were 54.2941, 53.5508, 49.2557, 53.5558, 53.1298, 54.09211, and 36.9567 at 1h, 6h, 24h, 48h, 72h, 7days, and 14 days, respectively showed in Figure (1).

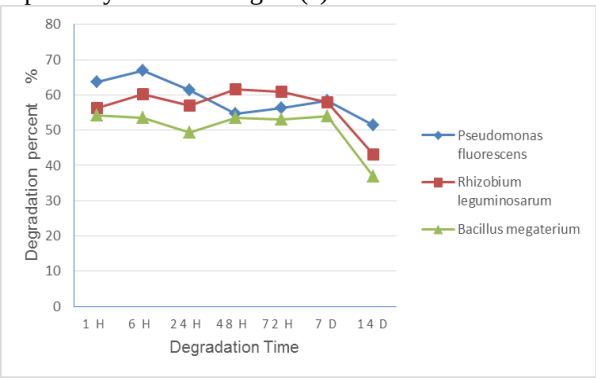

Figure 1. Efficiency of Bacterial strains on Chlorpyrifos Degradation.

Detection of Chlorpyrifos, TCP, and Chlorpyrifos Oxon by HPLC*:*

The present study reports the results of HPLC analysis of Chlorpyrifos (CPF) converting to 3,5,6-trichloro-2-pyridinol (TCP) and Chlorpyrifos Oxon (CPF-oxon) by *Pseudomonas fluorescens* and *Rhizobium leguminosarum* which are the two highly bacterial species as well as mixed strains incubated in LB medium for 14 days containing Chlorpyrifos pesticide as carbon source. All treatments were observed during the 14 days of exposure to Chlorpyrifos pesticide and which led to significant results. In this study, the chromatographic profiles obtained the high rates of Chlorpyrifos, TCP, and Chlorpyrifos Oxon under HPLC conditions. The retention times were 1.7 min for Chlorpyrifos, 1.0 min for Chlorpyrifos Oxon and 0.6 min for TCP. The total retention times were 5 minutes (Table 2).

Based on the HPLC results, the highest concentration of CPF was detected in the control sample treated with only Chlorpyrifos pesticide without adding any bacterial strains. The retention time was 1.7min (Figure. 2A).

In LB medium sample containing *Rhizobium leguminosarum*, HPLC results showed that the bacterial strain degraded CPF to CPF Oxon whose retention time was 1.001 (Figure 2B). On the other side, the LB medium sample containing *Pseudomonas fluorescens* bacteria, the Chlorpyrifos degraded to TCP whose retention time was 0.637 min (Figure 2C).

When mixing the two types of *Pseudomonas fluorescens* with *Rhizobium leguminosarum* strains in one LB medium sample, the hydrolysis result of chlorpyrifos detected by HPLC analysis was TCP compound. The retention time was 0.655 minutes (Figure 2D).

Table 2. Retention times, area, and heights of chlorpyrifos, chlorpyrifos oxion and TCP by incubation with *Pseudomonas fluorescens, Rhizobium leguminosarum* and *Pseudomonas fluorescens* with *Rhizobium leguminosarum* for 14 days in LB medium.

	Retention Time	Area	Height
Chlorpyrifos	1.71	3174.32	180.72
Rhizobium leguminosarum	1.001	8098.025	492.3599
Pseudomonas fluorescens	0.637	4957.507	524.4167
Rhizobium leguminosarum and Pseudomonas fluorescens	0.655	3154.051	321.105

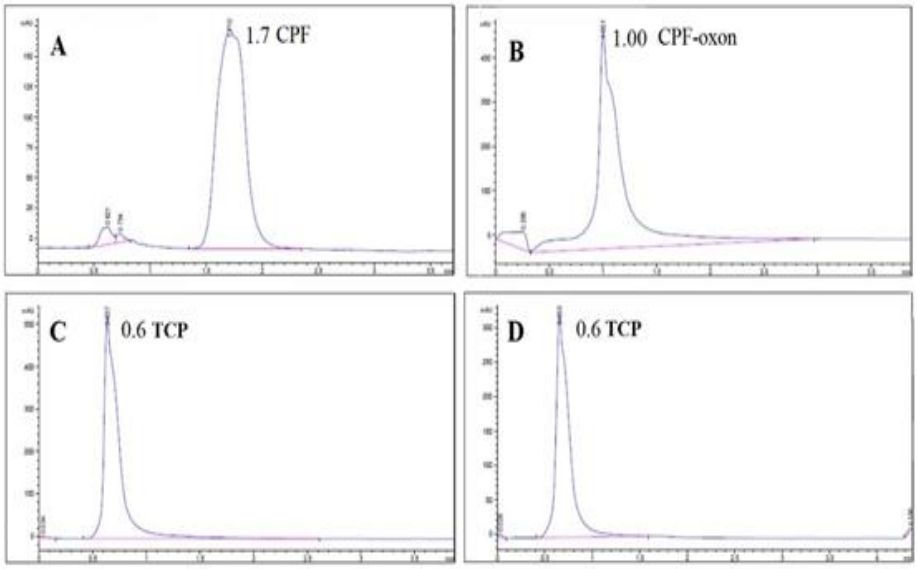

Figure 2. HPLC Chromatogram of Chlorpyrifos retention time in LB medium.
A: Chlorpyrifos
B: Degradation by *Rhizobium leguminosarum*.
C: Degradation by *Pseudomonas fluorescens*.
D: Degradation by *Pseudomonas fluorescens* with *Rhizobium leguminosarum*

Screening degradation genes in bacterial strains:

In this study, the expected lengths of PCR fragments are 700bp for F196 in *Bacillus megaterium*, 700bp for R840 in *Rhizobium leguminosarum*, 650bp for Mpd in *Bacillus megaterium*, 950bp for Opd in *Bacillus megaterium* and *Pseudomonas fluorescens*, 500bp for R757 in *Rhizobium leguminosarum*, 300bp for F450 in *Bacillus megaterium* and *Rhizobium leguminosarum* finally 500bp for 2350-2351 in *Rhizobium leguminosarum* shown in Figure (3)

The calculation of similarity coefficients was based on the amplified DNA bands shown in Table (3). The studied genotypes formed two main clusters; *Bacillus megaterium* and *Pseudomonas fluorescens* with similarity of gene degradation about 35%, while *Rhizobium leguminosarum* showed a different path in gene degradation (Figure 4).

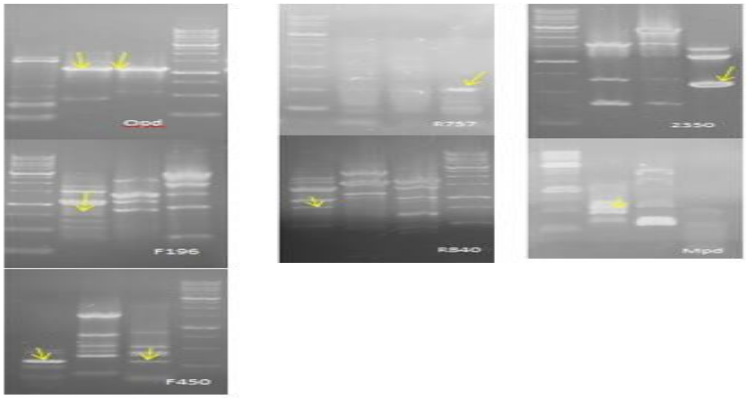

Figure 3. Screening of degradation genes by three bacteria A: *Bacillus megaterium*, B: *Pseudomonas fluorescens*, C: *Rhizobium leguminosarum*

Table 3. Diagram of degradation genes profiles generated by three bacteria A: *Bacillus megaterium*, B: *Pseudomonas fluorescens*, C: *Rhizobium leguminosarum*

primer	bp	A	B	C	primer	bp	A	B	C	primer	bp	A	B	C
	3000	0	0	1		2000	1	0	0		2500	0	1	0
	2200	0	1	0		1500	0	1	0		2400	0	0	1
	2000	0	0	1		1450	0	1	0		1700	0	0	1
	1700	1	0	0		850	1	0	0		1600	1	1	0
	1500	1	0	0		750	0	1	0		1500	0	0	1
	1400	1	1	1		550	0	1	0		1400	1	0	0
	1050	1	1	0		500	1	1	0		1300	0	1	0
F 196	950	0	0	1	OPh B	420	0	1	1	F450	1100	0	1	0
	900	0	1	1		400	1	0	0		1000	1	0	1
	850	1	1	1		390	0	0	1		750	0	0	1
	790	1	0	0		370	1	1	0		600	1	0	1
	750	1	0	1		300	1	0	1		500	0	0	1
	600	1	0	0		200	0	0	1		450	1	1	0
	530	1	0	0		150	0	1	0		400	1	0	0
	500	0	0	1		130	0	0	1		100	0	1	0
	400	0	0	1		100	1	0	0					
	2000	0	1	1		1600	1	0	0		2500	0	1	1
	1500	0	0	1		1400	0	1	0		2250	0	0	1
	1450	0	0	1		1000	0	1	0		2000	0	0	1
	1400	0	1	0		700	1	1	0		1750	1	1	1
	1150	1	1	0		600	0	1	0		1400	0	1	0
	950	1	1	0		500	1	1	1		1250	0	0	1
Opd	800	1	1	0	2350/2351	450	0	1	1	R 757	1200	0	1	0
	750	0	1	0		400	0	0	1		1000	1	0	0
	600	0	0	1		350	1	0	0		850	0	1	0
	550	1	0	0		300	0	0	1		700	1	0	0
	450	0	1	1		250	0	1	1		650	0	0	1
	400	0	0	1		200	1	0	0		600	1	0	0
	200	0	0	1		100	1	0	1		250	0	1	0
	2000	0	1	0		2000	1	1	0		2900	0	1	0
	1500	0	1	0		1400	0	1	0		2500	0	0	1
	650	1	0	0		1300	0	1	1		1500	1	1	0
	500	1	0	0		1000	0	1	1		1400	1	1	1
	500	0	1	0		850	0	0	1		1300	0	1	1
pd	400	1	0	1	R 840	800	1	0	0	R 977	700	1	1	0
	350	1	1	0		750	0	0	1		650	0	0	1
	300	0	1	1		700	0	0	1		400	1	1	0
	200	0	0	1		300	0	1	0					
	150	0	0	1		250	1	0	0					
	100	0	0	1										

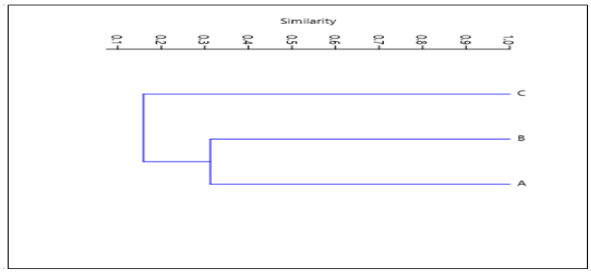

Figure 4. Dendrogram for the phylogenetics relationships among the three types of bacteria based on degradation genes data. A: *Bacillus megaterium*, B: *Pseudomonas fluorescens*, C: *Rhizobium leguminosarum*

Discussion

The degradation of pesticides by microorganisms is the primary mechanism of degradation and detoxification. Bacteria have the highest potential of growth on chlorpyrifos (substrate) among all microorganisms. Bacteria can degrade synthetic chemicals found in the environment and use them as source of carbon and energy. Chlorpyrifos can be used directly as a source of bacteria C, P, and N (Singh *et al.*, 2004 and Awad *et al.*, 2011). Accordingly, this study showed the ability of some bacterial genera to degrade the pesticide chlorpyrifos. It was reported that 75–87% degradation of chlorpyrifos has been done by a lot of bacterial genera including *Pseudomonas fluorescens* (Lakshmi, *et al.*, 2008).

Oxidative sulfur is removed from chlorpyrifos by cytochrome(s) P-450 resulting in the formation of the unstable chlorpyrifos-oxone intermediate that abruptly degraded to TCP and DETP. TCP contains a pyridinol ring with three chlorine atoms attached to it. In this study, HPLC analysis showed the degradation of Chlorpyrifos pesticides to TCP and Chlorpyrifos Oxon by *Pseudomonas fluorescens* and *Rhizobium leguminosarum* incubated in LB medium within 14 days (Xu *et al.,* 2008). It is reported that TCP can be used as the source of carbon and energy by *Pseudomonas* (Racke, 1993). *Pseudomonas sp.* has been reported to degrade OP compounds catabolically as C-, N- or P-sources or co-metabolically (Bano and Musarrat, 2003; Cycoń *et al*., 2009) (Figure5).

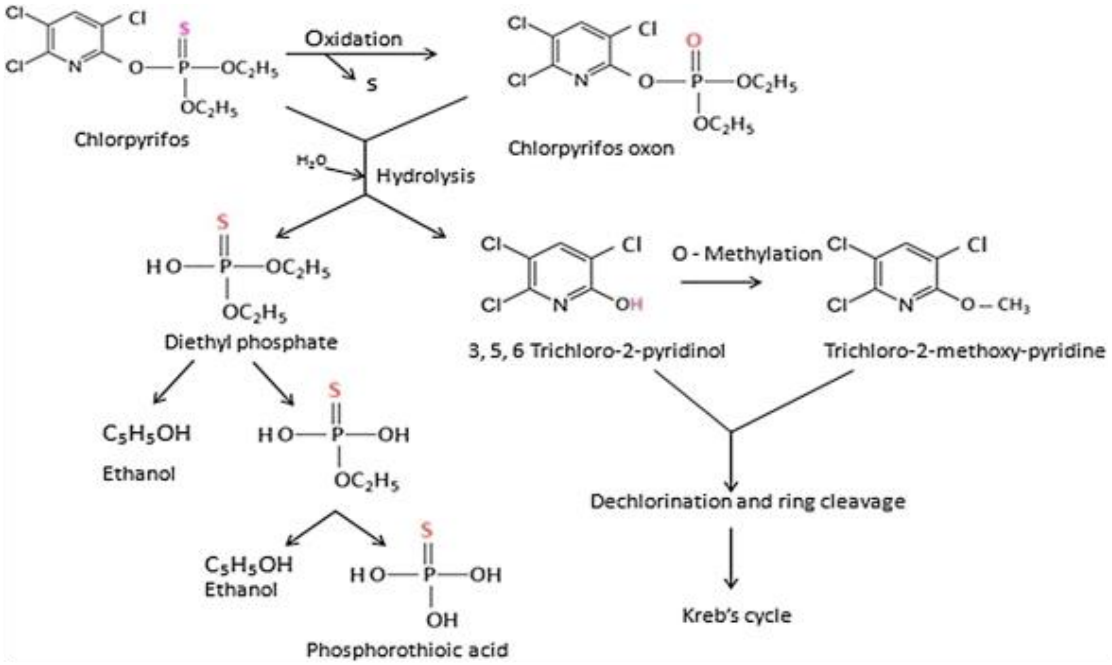

Figure 5. Schemes for the degradation pathways of Chlorpyrifos. (Singh *et al*., 2004)

The process of degradation of Chlorpyrifos to TCP was observed in *Pseudomonas fluorescens* bacteria sample, which indicates that *Pseudomonas fluorescens* uses the carbon contained in Chlorpyrifos and converts Chlorpyrifos to TCP. It was found that *Rhizobium leguminosarum* was able to hydrolyze chlorpyrifos in LB medium to Chlorpyrifos Oxon. Similar results were obtained in another study where TCP formation did not occur directly during CPF degradation by Rhizobium species. In contrast, during CPF degradation by *Rhizobium* sp., the TCP configuration does not appear directly in the media samples. It is suggested that this bacterial strain may utilize some alternative mechanisms of CPF degradation (Lakshmi *et al.* 2008).

Chlorpyrifos was observed to be degraded to TCP in the sample that contains the two types of bacteria together. Possibly *Pseudomonas fluorescens* did the catabolism directly, or *Rhizobium leguminosarum* decomposed chlorpyrifos to Chlorpyrifos oxon and then *P. fluorescens* converted Chlorpyrifos oxon to TCP.

The results of Chlorpyrifos biodegradation in LB Medium have demonstrated that all three bacterial isolates were able to degrade Chlorpyrifos based on using one concentrate through the 14 days. *Pseudomonas fluorescens* had the highest efficiency to degrade Chlorpyrifos pesticide with 58.90 %, then *Rhizobium leguminosarum* with 56.72% and finally *Bacillus megaterium* strain with 50.69 %. In this study, significant bacterial cell growth in the three bacterial strains was detected in LB medium containing Chlorpyrifos as the carbon source (Richins *et al.* 1997). Chlorpyrifos has also been reported to be degraded by bacterial genera, which needs extra carbon sources (Richins *et al.* 1997, Mallick *et al.* 1999).

The Chlorpyrifos percent of degradation in LB medium was supplemented with 1.5 ml/L Chlorpyrifos showed in Figure. (1). It was observed that the percent of Chlorpyrifos degradation for these three bacterial isolates were different at the same incubation period. It has been found that the percent of Chlorpyrifos degradation by the isolates *Pseudomonas fluorescens*, *Rhizobium leguminosarum*, and *Bacillus megaterium* were greatly increased (Li *et al.* 2008; Xu *et al.* 2008). They also showed that 1.5 ml/L Chlorpyrifos was degraded to a detectable level in the 14 days by *Pseudomonads flurescence* strain belonging *Pseudomonas* sp. The percent of Chlorpyrifos biodegradation by *Pseudomonas fluorescens* and *Bacillus megaterium* on the 7th day of the incubation period were 58 and 54, respectively, nearly similar to Lakshmi *et al.* (2008) which were 43, 45 after 10 days of incubation by *Pseudomonas fluorescence and Bacillus melitensis*. Some studies presented a lot of information about the degradation rates of Chlorpyrifos by the different species of *Pseudomonas* (Li *et al*., 2008; Xu *et al.,* 2008).

Previous reports regarding degradation of chlorpyrifos efficiently via genetic determinants were suggested that *E. coli* and *P. fluorescens* bacterial isolates inhabiting different ecosystems, and provide strong the basis for the development of bioremediation strategies in the area (Murtaza *et al*., 2018). Numerous studies have developed primers that can be used for the detection of organophosphates degradation genes. Those degradation

genes have been isolated and studied from different species with different activities. Opd and mpd genes appear to be indifferent bacterial strains as Opd genes have been found in *Agrobacterium radiobacter, Flavobacterium sp, Pseudomonas diminuta, pseudomonas putida, plesiomonas species, Sphingomonas sp* (Zhongli *et al.,* 2001; Horne *et al.,* 2002; and Iyer *et al.,* 2013). While, mpd gene was detected and isolated from Plesiomonas *sp., Sphingomonas sp., Pseudomonas sp., Stenotrophomonas sp.,* (Cui *et al.,* 2001; Li *et al.,* 2007; Liu *et al.,* 2005; Yang *et al.,* 2006 and Abdullah *et al.,* 2016). PCR has been put to work for genotyping bacteria quickly and accurately according to degradation genes. The results in this study proved that the three types of bacteria are related to each other through degradation genes, as *Bacillus megaterium* and *Pseudomonas fluorescens* have the same genetic similarity for these genes, but *Rhizobium leguminosarum* has another pathway in genetic similarity, therefore we believe that this may be the reason for changing the degradation pathway for these bacteria individually.

In this study, the bacterial isolates showed great biodegradation for Chlorpyrifos pesticide in LB medium. All three bacterial strains introduced significant results for the degradation but, *Pseudomonas florescence* and *Rhizobium Leguminosarum* strains were the highest in the degradation process which analyzed the Chlorpyrifos component to TCP and Chlorpyrifos Oxon which have a little toxic effect on the ecosystem. As well as, the results encourage recommending using the previous strains to reduce the Chlorpyrifos toxicity in the environment especially in the soil and plants.

Compliance with ethical standards Conflict of interest: The authors declare that they have no conflict of interest. **Ethical approval:** This article does not contain any studies with human participants or animals performed by any of the authors.

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التحلل الحيوي للكلوربيريفوس بواسطة بعض السلالات البكتيرية وفحص جينات التحطيم بها

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التحلل الحيوي هو عملية طبيعية ، حيث يكون تدهور مادة كيميائية أو مبيدات الآفات الزنوبوتية من قبل كائن حي هو في المقام الأول استراتيجية لبقائها على قيد الحياة. وتتدهور مبيدات الآفات في البيئة أساسا بفعل الكائنات الحية الدقيقة. تهدف الدراسة الى تقييم قدرة ثلاث سلالات بكتيرية على تكسير الكلوربيريفوس، وتحديد الجينات المسؤولة عن هذا التحلل ، ودراسة طريقة التحلل الكيميائية الحيوية الخاصة بهم. تم الكشف عن ثلاث سلالات بكتيرية *Pseudomonas fluorescens, Rhizobium leguminosarum,* *Bacillus megaterium* في بيئة (LB) تحتوي على الكلوربيريفوس ووجد أن *P. fluorescens* كانت الأعلى كفاءة 58.90٪، ثم R. *leguminosarum* 56.72٪، وأخيرا *B. megaterium* بكفائة تحلل تقدر بـ 50.69٪. ووفقا للتشابه الجيني لجينات التدهور وجد تشابة بين كلا السلالتين البكتيرتين *B. megaterium* and *P. fluorescens* ، في حين أن *R. leguminosarum* مختلفة في مسارها. وكانت سلالات *P. florescence* و *R. Leguminosarum* هي الأعلى في عملية التحلل التي حللت مكون الكلوربيريفوس إلى 3,5,6-trichloro-2-pyridinol (TCP) و Chlorpyrifos Oxon الذي له تأثير سام قليلا على النظام البيئي. مع النتائج التي تم الحصول عليها يوصى بتطبيق هذه السلالات على التربة والنباتات للحد من سمية الكلوربيريفوس في البيئة.