ORIGINAL RESEARCH PAPER

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KEY WORDS: Enrichment culture, Malathion biodegradation and 16S rRNA

MICROBIAL DEGRADATION OF ORGANOPHOSPHORUS COMPOUNDS IN EGYPTIAN AGRICULTURE SOIL.

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ABSTRACT

Enrichment culture technique was employed to isolate different bacterial strains which are capable of Malathion degradation in agriculture soil of Abiess district, Alexandria government, Egypt. Six bacterial strains were identified; *Pseudomonas aeruginosa* strain DBT1BNH3, *Rhodococcus* sp. strain L9, *Bacillus licheniformis* strain CAS20, *Bacillus aerius* strain CH2-D42-30, *Bacillus* sp. (in: Bacteria) strain lzp02, and *Bacillus safensis* strain APBSDSB46.Identification was done using morphological, biochemical, and 16S rRNA sequence alignments methods. All six isolates were able to grow in mineral salt medium supplemented with Malathion (20 mg/l) as a sole carbon source. High performance liquid chromatography was done to determine the residues of Malathion quantitatively within different time intervals (first, seventh, fourteen days of exposure); results ranged from 44.8% to 87.9% for *Bacillus safensis* strain APBSDSB46and *Pseudomonas aeruginosa* strain DBT1BNH3 respectively. In addition, Carboxylesterase (CE) gene was detected for six bacterial isolates with varied DNA content.

INTRODUCTION

Recently, Organophosphorus compounds considered as the most dominant applicable pesticides, accounting for more than 38 % of total world pesticide market (Theriot and Grunden 2011). This may be due to their broad-spectrum effectiveness against varied outdoor insects in both agricultural and residential settings (Newton et al.,1989).Among different Organophosphorus pesticides groups, malathion (O,O-dimethyl dithiophosphate) considered as the most intensively applicable agricultural insecticide (Cho et al., 2002).

U.S. Environmental Protection Agency (EPA) had categorized malathion as class III pesticide relaying on its toxicity influence on human beings; including kidney, liver, brain and lung damages(Ray and Richards 2001and Kumar et al, 1996). Furthermore, malathion had been categorized as a carcinogenic agent causing human blood cells chromosomal aberrations, gene loss, and general DNA damage (Kanade et al., 2012). The maximum allowed malathion residue on crops used as food is only 8 parts per million (U.S. EPA. Office of Pesticide Programs 1988). Malaoxonwhich is considered as thehighest toxic metabolite of malathion can spread easily in air (WHO 1997), causing annually about 200,000 deaths, and more than 3 million poisoning cases. (Karalliedde and Senanayake, 1988; Sogorb et al., 2004).Malathion binds to the enzyme acetylcholinesterase (AChE) at nerve endings throughout the bodies of insects and other organisms, resulting in accumulation of acetylcholine at the nerve junctionand overstimulation of the nervous system. (Reigart and Roberts, 1999).

Different physical, chemical, or biological methods could be employed to degrade or detoxify malathion. Comparing in-situ bioremediation methods, microorganism'sbiodegradation is consideredthe safest and most economic natural process to degrade active pesticides ingredients(Sassman*et al*., 2004; Nawab *et al*., 2003) (Chapalamadugu and Chaudhry 1992; Singh and Walker 2006). Various bacterial and fungal species have been recorded with capability of growing on malathion such as *Serratia* sp. and *Pseudomonas* sp. (Cycon et al., 2009), *Providenciastuartii* MS09 (Rani et al., 2008), *Agrobacterium* sp. (Horne et al., 2002), *Paracoccus* sp. strain TRP (Xu et al., 2008), *Entrobacter* strain B-14 (Singh et al., 2004), *Arthrobacter* sp. (Racke, 1993), *P. putida* (Goda et al., 2010), *Aspergillus* sp. and *Penicillium* sp. (Ningfeng et al., 2004). Bacterial carboxylesterase enzymeshydrolyze carboxylic ester bonds found in malathion, with relatively substrate specificity, and high sequence similarity (Jakoby and Ziegler 1990). This investigation aimed to identify Egyptian bacterial isolates with different capability for malathion biodegradation with biochemical and molecular markers methods.Then, carboxylesterase gene which consider the main malathion biodegradation key role was studied and compared via sequencing level among different bacterial isolates.

MATERIALS AND METHODS

Malathion

Malathion diethyl (dimethoxy thiophosphorylthio-succinate) was obtained from Sigma Company (PubChem Substance ID 32975529).

Sampling of Malathion degradation bacteria

Twenty soil samples were collected from completely separated agricultural locations from October 2018 to December 2018, Abiess district, Alexandria government, Egypt. After removing 5 cm of surfacing soil and 15 cm in depth, agricultural soil samples were collected and refrigerated at 4°C till use. After dissolving Organophosphorus compound, Malathion (MAL) in methanol, MF-Millipore™ Membrane Filter, 0.22 µm pore size (MERCK) was applied. Ten grams from each contaminated soil samples were added to 250 ml of minimal salt medium (MSM) supplemented with MAL (20 mg/l) as a sole carbon source and incubated for 14 days at 30°C in a rotary shaker (CS-NOR,Cleaver Scientific UK) at 200rpm/1rpm. After that, MSM plates which supplemented with 100 mg/l of MAL were inoculated with enrichment culture. Finally, single colonies were selected after incubation for 3 days at 300C, and identified according to morphological, biochemical and molecular characterization (Saafan et al., 2016).

Biochemical and molecular Bacterial isolates characterization

Bergry's manual was applied for bacterial biochemical identification according to Holt *et al.,* (2012). Molecular characterization was represented by 16S rRNA gene which performed as molecular confirmatory marker (Yang et al., 2006). Total genomic DNA was purified through E.Z.N.A.® Bacterial DNA Kit (Omega Bio-TEK, USA) according to manufacturer protocol. Universal 16S rRNA primers 27f (5-AGAGTTTGATCMTGGCTCAG-3) and 1525r (5-AAGGAGGTGWTCCARCC-3) (Macrogen, Korea) were amplified specific PCR with approximately 1500 base pair (bp). In total volume of 25 µl, Dream Taq Green PCR Master Mix (2X) (Thermo Fisher Scientific Inc., USA) was used in presence of 10-20 ng of template DNA and 30 pmol of each primer. Thermal cycler conditions was constructed according to (Ye et al., 2012). Amplicons were visualized with gel electrophoresis using 1.5% (w/v) agarose gel and documented via Gel documentation system (Geldoc-it, UVP, England) supported by Totallab analysis software, ww.totallab.com, (Ver.1.0.1). Resultant RT-PCR products were purified with Micro spin filters and quantities spectrophotometrically. Sequence analysis was employed using the ABI PRISM® 3100 Genetic Analyzer (Micron-Corp.Korea). Aligned sequences were analyzed on NCBI website (http://www.ncbi.nlm.nih.gov/webcite) using BLAST to confirm their identity. ClusteralW software analysis (www.ClusteralW.

com) was applied for constructing neighbor-joining phylogenetic trees

Bacterial Biodegradation assay

Dilution plate count technique was applied for quantifying colony forming units per milliliter (CFU/ ml) of bacterial isolates and inoculated two hundred milliliter of MSM supplemented with 200 mg/l of MAL 3.0 ×108 CFU/ml and incubated at 30°C with shaking at 150 rpm. Serial exposer duration (1, 7 and 14 d from inoculation with negative control (uninoculated medium) were designed to evaluate Malathion biodegradation. Double extraction with 50 ml dichloromethane was carried out for centrifuged ten milliliters of previous media to extract Malathion. After passing through anhydrous Na2SO3, dichloromethane sample were evaporated through rotary evaporator at 30°C. Acetonitrile was applied to dissolve dry residue. HPLC (Agilent Technologies 1260 Infinity, USA) with Eclipse plus C18 (4.6 ×250mm 5 µm) and diode array detector (DAD) at λ 210 nm was used with Acetonitrile: water (70:30), at a flow rate 1 ml/min.

Mining of carboxylesterase enzyme for Organophosphorus biodegradation

To get better understanding for Organophosphorus biodegradation mechanism, carboxylesterase gene was mined and studied at sequencing level for different bacterial isolates. Specific primer CE-f (5 -GAC GCC TGT GTG ATC TGG TTG-3) and CE-r (5 -CCC AGG TGT TGA GGT ACT CGA-3 was applied to amplify ~ 500 bp of carboxylesterase gene portion. Thermal cycler condition as follow, denaturation for 4 min at 95C; then 30 cycles consisting of 94C for 1 min for denaturation, 62C for 1 min for annealing, 72C for 1 min for extension and a final extension step at 72C for 7 min (Goda et al., 2010).

RESULTS

Malathion biodegradation bacterial identification:

Twenty soil samples contaminated with different pesticides collected from Abiess district, Alexandria government, Egypt were applied to isolate Malathion pesticide biodegradation organisms through sub culturing on MSM liquid medium supplemented with 20 mg/l of MAL. different biodegradation isolates were identified via morphological and biochemical and molecular features. Interestingly, only six different bacterial isolates reflected different malathion biodegradation abilities. In the light of biochemical characterization, biodegradable bacterial isolates could be identified as *Pseudomonas, Rhodococcus*and *Bacillus* for first, second and third, fourth; fifth isolates respectively (Table 1).

Table (1): morphological and biochemical bacterial identification.

		Degradable selected isolates					
		First	*Second*	*Third*	*Fourth*	*Fifth*	*Sixth*
Morphological character	shape	Rods	Rods	Rods	Rods	Rods	Rods
Biochemical character	Gram stain	Negative	(+ve)	(+ve)	(+ve)	(+ve)	(+ve)
	Catalase	(+ve)	(+ve)	(+ve)	(+ve)	(+ve)	(+ve)
	Gelatin liquefaction	(+ve)	(+ve)	(+ve)	(+ve)	(+ve)	(+ve)
	Citrate utilization test	(+ve)	(+ve)	(+ve)	(+ve)	(+ve)	(+ve)
	Urease test	(-ve)	(+ve)	(-ve)	(-ve)	(-ve)	(-ve)
	Indole test	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)	(-ve)

Evaluation Malathion Bacterial biodegradation ability

Malathion biodegradation ability was assays through incubate 20 mg/l of malathion as a sole carbon source with five identified bacterial isolates for 1, 2, 4, 7, 11 and 14 days. Degradation activity was recorded and monitored via HPLC method. As shown by table (2) and Figure (1) except *Pseudomonas sp* which degraded 9.8% of malathion, no detectable biodegradation activity was recorded for all bacterial isolates after incubation with malathion for 24 hours. Then different biodegradation activities were detected after 7 days of incubation. Third *Bacillus*sp*was superior* for malathion degradation (43.6 %) comparing with second *Bacillus sp*which only degrade 25.5% of malathion. After 14 days of incubation, 87.9 % of malathion was degraded through *Pseudomonas* sp. on the other hand, 44.8% of Malathion was degraded within 14 days by fourth *Bacillus sp.*

Malathiondegradationcapabilitywasdescendinglyas*Rhodococcuss p,* first, second, third and fourth *Bacillus*sp isolates of 71.1, 53.2, 50.3, 48.4 and 44.8 % respectively.

On the other hand, Goda et al. (2010) indicated that MAL degradation in the presence of other carbon sources was relatively lower than when MAL was present as a sole carbon source for the first 2 days and after 4 days of the degradation was the same in both cases. By contrary of our Malathion application experiment, 78% of the Malathion initially added to the medium was hydrolyzed by the strain ML-1 within 5 days of incubation time (Khan et al., 2016). Different Malathion degradation patterns were recorded by Singh et al., (2013). They found that 74.11% of malaoxon (an analog of Malathion) was degraded by *Bacillus* sp. in accordance by our findings, Reverse phase HPLC-UV analysis indicated degrade 36.22% and 49.31% of Malathion, respectively, after 7 days of incubation through *Bacillus Brevibacillus* sp. and *Bacillus cereus* in liquid media (Singh et al. 2012).

Table (2): Malathion degradation percentage of three bacterial isolates for 1, 7 and 14 exposer days.

Bacterial isolates	Biodegradation activity (%)		
	Malathion exposer days.		
	1	7	14
Pseudomonas sp.	9.8	42.7	87.9
Rhodococcus sp.	0	31.8	71.1
Bacillus sp.	0	28.7	53.2
Bacillus sp.	0	25.5	50.3
Bacillus sp.	0	43.6	48.4
Bacillus sp.	0	40.8	44.8

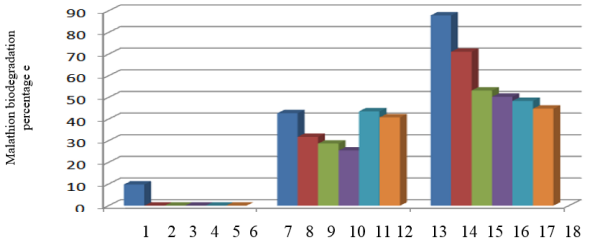


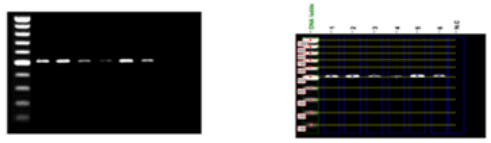
Figure (1): Malathion degradation percentage of three bacterial isolates for 1, 7 and 14 exposer days.

1- *Pseudomonas sp for 1 day of Malathion exposer.*	2- *Rhodococcus sp. for 1 day of Malathion exposer.*
3- *First Bacillus sp. for 1 day of Malathion exposer.*	4- *Second Bacillus sp. for 1 day of Malathion exposer.*
5- *Third Bacillus sp. for 1 day of Malathion exposer.*	6- *Fourth Bacillus sp. for 1 day of Malathion exposer.*
7- *Pseudomonas sp for 7 day of Malathion exposer.*	8- *Rhodococcus sp. for 7 day of Malathion exposer.*
9- *First Bacillus sp. for 7 day of Malathion exposer.*	10- *Second Bacillus sp. for 7 day of Malathion exposer.*
11- *Third Bacillus sp. for 7 day of Malathion exposer.*	12- *Fourth Bacillus sp. for 7 day of Malathion exposer.*
13- *Pseudomonas sp for 7 day of Malathion exposer.*	14- *Rhodococcus sp. for 7 day of Malathion exposer.*
15- *First Bacillus sp. for 7 day of Malathion exposer.*	16- *Second Bacillus sp. for 7 day of Malathion exposer.*
17- *Third Bacillus sp. for 7 day of Malathion exposer.*	18- *Fourth Bacillus sp. for 7 day of Malathion exposer.*

16S rRNA gene sequences identification

PCR products for 16S rRNA genes of six bacterial isolates were amplified, eluted, sequenced and alignments via Blast program (photograph, 1). As shown by Figure (2) and Table (3), data alignments reflected that, first specific 16S rRNA fragment sequence was 100 % similarity to that of *Pseudomonas aeruginosa* strain DBT1BNH3 (GenBank accession no FJ976651.1). Additionally, second specific 16S rRNA gene fragment sequence showed 100 % of similarity to of *Rhodococcus* sp. strain L9 (GenBank accession no MK183003.1). interestingly, third, fourth, fifth and sixth 16S rRNA fragment sequences were similarity to that of *Bacilluslicheniformis* strain CAS20, *Bacillusaerius* strain CH2-D42-30, *Bacillus* sp. (in: Bacteria) strain lzp02 and *Bacillussafensis* strain APBSDSB46 with 100, 100, 100 and 98 % identity percentages respectively. Six obtained bacterial isolates were selected with highly similarity isolates to construct phylogenetic tree based on 16S rRNA sequences (Figure 3). Several *Pseudomonas* and*Bacillus* species have already been reported to degrade different OP compounds, including malathion. With accordance of our findings, Mohamed *et al.* (2010) identified and assayed *Bacillus thuringiensis*for utilize malathion as sole carbon and phosphorus source. As similar, Hamouda*et al.* (2013) isolated nine malathion-degrading bacterial strains belonging to *Acinetobacter* and *Bacillus* species. More support to our findings was added by Singh et al., 2012. Theyemployed enrichment culture technique isolated *Brevibacillus* sp. and *Bacillus cereus*malathion-degrading bacterial strains from an agricultural soil sample.

Ph. (1): 16S rRNA specific genomic products (~1400 bp) (A) and computerize analysis (B) for (A), (B), (C), (D) and (D), (E) (first, second, third, fourth, fifth and sixth bacterial isolates respectively).

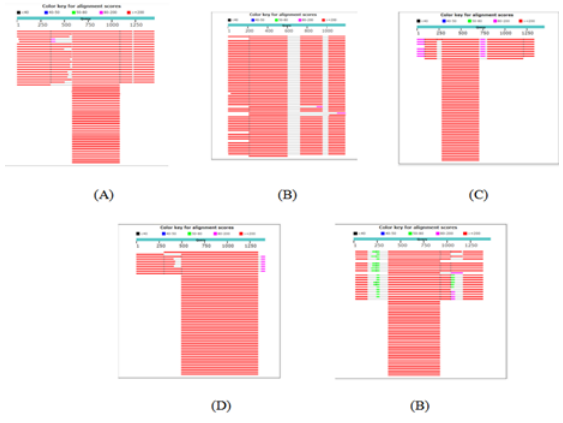


(A) (B)

1 (2)	2 (3)	3 (4)	4 (5)	5 (6)	6 (7)
Lane %	Lane %	Lane %	Lane %	Lane %	Lane %
1.78	1.68	1.25	1.12	1.56	1.05

(C)

Figure (2):16S rRNA sequence and alignment data for (A), (B), (C), (D) and (D), (E)(first, second, third, fourth, fifth and sixth bacterial isolates respectively).



(A) (B) (C) (D) (B)

Table (3): Molecular identification of 16S rRNA gene partial sequence for oily sludge bacteria.

Isolates	Identification	Accession number	Identity (%)
1	*Pseudomonas aeruginosa* strain DBT1BNH3 16S ribosomal RNA gene, partial sequence	FJ976651.1	100
2	*Rhodococcus* sp. strain L9 16S ribosomal RNA gene, partial sequence	MK183003.1	100
3	*Bacilluslicheniformis* strain CAS20 16S ribosomal RNA gene, complete sequence	KY199559.1	100
4	*Bacillusaerius strain* CH2-D42-30 16S ribosomal RNA gene, partial sequence	MK287636.1	100
5	*Bacillus* sp. (in: Bacteria) strain lzp02 16S ribosomal RNA gene, partial sequence	MH071746.1	100
6	*Bacillussafensis* strain APBSDSB46 16S ribosomal RNA gene, partial sequence	MG705592.1	98

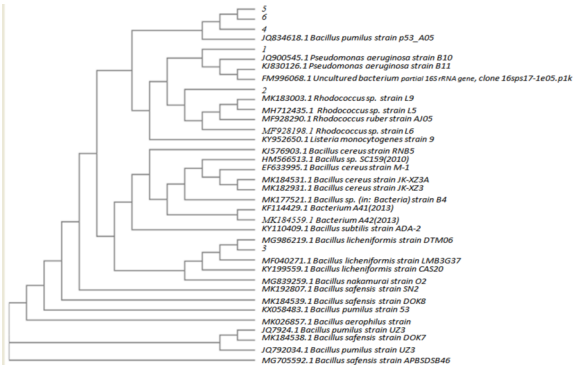
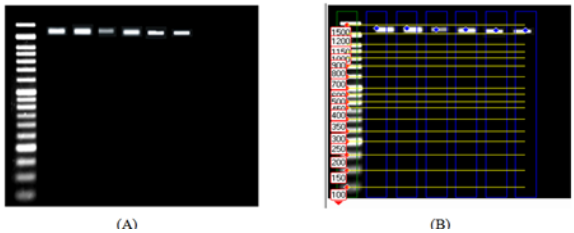


Figure (3): phyllogenetic tree for six bacterial isolate based on 16S rRNA gene sequence.

Carboxylesterase (CE) gene amplification

Carboxylesterase (CE) gene which considers the key role for malathion degradation was amplified via six bacterial isolates with different malathion biodegradation capability. Different carboxylesterase (CE) gene genomic content was evaluated for each of bacterial isolates. First bacterial isolates which identified as *Pseudomonas aeruginosa* strain DBT1BNH3 reflected highest DNA content for carboxylesterase (CE) gene (1.78 %). By contrary, lowest carboxylesterase (CE) DNA content was recorded for sixth bacterial isolates which identified as *Bacillussafensis* strain APBSDSB46 (1.05%). Second, fifth, third and fourth bacterial isolates which identified as *Rhodococcus* sp. strain L9, *Bacillus* sp. (in: Bacteria) strain lzp02, *Bacilluslicheniformis* strain CAS20 and *Bacillusaerius* strain CH2-D42-30 showed 1.68, 1.56, 1.25 and 1.12 % of DNA content for carboxylesterase (CE) gene respectively. Our findings for employing PCR method to amplifycarboxylesterase gene with specific primers CE-F and CE-Rwas in accordance of (Godae*t al.,* 2010), theyapplied the same method and primers to detect carboxylesterase gene for different malathion-degrading bacterial strains. More light was added to our results for using carboxylesterase gene as specific marker by Xie et al., (2013), as they cloned amplified carboxylesterase gene from *A. tengchongensis*into *E. coli* BL21 (De3).



(A) (B)

Ph. (2): Carboxylesterase (CE) gene products (~500 bp) (A), computerize analysis (B) and DNA content (C) for first, second, third, fourth, fifth and sixth bacterial isolates.

CONCLUSION

In the present study, six bacterial isolates that capable for efficient degradation of the organophosphorus insecticide Malathion were identified via morphological, biochemical and molecular methods.

six bacterial isolates 16S rRNA specific products were amplified, sequenced, and identified as *Pseudomonas aeruginosa* strain DBT1BNH3, *Rhodococcus* sp. strain L9, *Bacilluslicheniformis* strain CAS20, *Bacillusaerius* strain CH2-D42-30 *Bacillus* sp. (in: Bacteria) strain lzp02,and *Bacillussafensis* strain APBSDSB46. Different Malathion biodegradation responses were recorded for different bacterial isolates with via 1, 7 and 14 exposer days. Furthermore, Carboxylesterase (CE) gene were mined among six bacterial isolates and detected with different genomic content.

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