Journal of Environmental Science and Technology

ISSN 1994-7887

science alert

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OPEN ACCESS Journal of Environmental Science and Technology

ISSN 1994-7887
DOI: 10.3923/jest.2018.175.189

Research Article
Identification of Gene Encoding Organophosphorus Hydrolase (OPH) Enzyme in Potent Organophosphates-degrading Bacterial Isolates

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Abstract

Background and Objective: The wide and indiscriminate use of pesticides for pest control in agriculture has inflicted serious harm and problems to humans as well as to the biodiversity. Microbial biodegradation of pesticides in contaminated soils has been considered advantageous to decontaminate areas that have been polluted by pesticides. The aim of this study consisted in isolation and molecular identification of potent bacterial isolates in pesticides biodegradation. **Materials and Methods:** The PCR amplification of 16sr DNA and *Gro*El genes were used to identify the potent bacterial isolates. The ability of bacterial isolates in pesticides biodegradation by gas chromatography was investigated. Detection and sequencing of *oph* gene in the bacterial isolates were employed. The enzymatically- generated degradation products of pesticides by GC/MS were detected. **Results:** Organophosphorus pesticides, diazinon and chlorpyrifos were the most persistent pesticides in the Egyptian soils samples. Four bacterial isolates GH10, GH2NO8, GH9OP and GH4SNO/P was identified as *Cronobacter muytjensii* strain GH10, *Pseudomonas aeruginosa* strain GH2NO8, *Achromobacter xylosoxidans* strain GH9OP and *Pseudomonas putidas* train GH4SNO/P, respectively based on 16s rDNA and *Gro*El gene sequence analysis. They were able to degrade 92.9, 91.82, 97.75 and 90.78% of diazinon at initial concentration (600 mg L^{-1}) as compared to 16.99% in control and 93.43, 78.51, 93.18 and 95.36% biodegradation of chlorpyrifos at initial concentration (480 mg L^{-1}) as compared to 4.28% in control, respectively, after 20 days. New organophosphorus hydrolase (*oph*) gene responsible for organophosphorus pesticides biodegradation was detected and sequenced in *Cronobacter muytjensii* strain GH10, *Pseudomonas aeruginosa* strain GH2NO8, *Achromobacter xylosoxidans* strain GH9OP, these sequences were deposited in GenBank under the accession number MF443872, MF443870 and MF443871, respectively. 3,5,6-trichloro-2-pyridinol (TCP) as metabolite of chlorpyrifos degradation and isopropyl-4-methyl-6-hydroxypyrimidine (IMHP) as metabolite of diazinon which were further metabolized to unknown polar metabolites was detected by GC/MS after 20 days in case of *Cronobacter muytjensii* strain GH10. **Conclusion:** The results of this study indicate that bacterial biodegradation is advantageous approach for pesticides bioremediation.

Key words: Organophosphates biodegradation, degrading-bacterial isolates, 16s rDNA, *Gro*El, *oph* gene, metabolic products

Citation: Ghada. M. El-Sayed, Nivien. A. Abosereh, S. A. Ibrahim, A. B. AbdEl-Razik, M.A. Hammad and Fatma. M. Hafez, 2018. Identification of gene encoding organophosphorus hydrolase (OPH) enzyme in potent organophosphates–degrading bacterial isolates. J. Environ. Sci. Technol., 11: 175-189.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The pesticides extensive use through accidental spills, handling, crop spraying, rinsing of containers, etc., has a potential to contaminate soil, air and water[1,2]. About 38% of total global pesticides consumption is accounted by organophosphorus pesticides which replaced organochlorines to a greater extent against crop loss by pest attack and improving crop yield[3].

Although (Ops) play important roles in protecting agriculture crop from weeds, insect pests and in disease-transmitting vectors controlling, they irreversibly inactivate acetyl choline esterase (AChE) which is essential to nerve function in insects, human and many other animals. Inhibition of acetyl choline esterase results in neurotransmitter acetyl choline accumulation and acetyl choline receptor continued stimulation thus, they could cause acute or chronic poising in human beings[4]. Chemical treatments, incineration and landfills are the current methods in OPs detoxification[5]. Incineration although is considered the most reliable method for OPs destructions has involved a serious public opposition due to the potentially toxic emissions[6]. Chemical methods are problematic due to production of large volumes of acids and alkali subsequently must be disposed. Landfills function adequately but leaching of pesticides into ground water supplies and surrounding soil is a big issue of concern.

One of the main methods to resolve the problems of (OPs) residues accumulation is to bioremediate these compounds[7,8]. The OPs contain three phosphoester bonds and hence are often termed phosphotriester. Hydrolysis of only one phosphoester bond (P-O, P-F, P-S and P-CN) can reduce significantly the toxicity of OPs by utilizing microbial enzymes that is an effective approach to degrade harmful OP compounds[9]. For example the hydrolysis of parathion resulted in 100-fold reduction in toxicity[10].

Various organophosphorus pesticides degrading bacteria able to use OPs as carbon, nitrogen or energy source were isolated from pesticides contaminated environments[11]. Pesticide degrading bacteria are including *Flavobacterium* sp.[12], *Pseudomonas diminuta* MG[13], *Penicillium lilacinum* BP[14] and *Arthobacter* sp.[15]. These bacterial strains have the ability to degrade OPs by different types of enzymes. The most characterized enzymes are phosphotriesterase (PTEs).

Phosphotriesterase (PTEs) are a group of enzymes that can degrade OPs, they are present in animals, plants and micro-organisms. Organophosphorus hydrolase enzyme (OPH) is a member of the amidohydrolase superfamily and encoded by *opd* gene. The bacterial *opd* gene has similar DNA sequence in different bacteria[16]. Methyl parathion hydrolase (MPH) is a member of β-lactamase superfamily. It is active against several OP compounds and is present in several phylogenetically unrelated bacteria[17].

Organophosphorus pesticides are commonly used in Egypt. Some bacterial strains have ability to convert these pesticides into sulfons or oxons or some other degradation products which may be less toxic than the parent molecule[18].

The goals of this study were to isolate and identify new bacterial strains from Egyptian agriculture soil able to degrade OPs insecticides as a sole source of carbon. The new isolates from this work may be better suited to the climate and environment conditions in Egypt. In present study, a novel gene *oph* encoding organophosphorus hydrolase was identified in *pseudomonas aeruginosa*, *Achromobacter xylosoxidans* and *Cronobacter muytjensii* for the first time.

MATERIALS AND METHODS

This study was conducted in early 2015, starting from January, 2015 until December, 2017.

Detection the most persistent pesticides in the Egyptians soils: Soil samples from agriculture fields in different governorates, Giza, Sharqia, Beheira, Kaliopia and Monofia, Egypt were isolated for the purpose of detection of the most persistent pesticides residues by Gas Chromatography analysis in the Central Agriculture Pesticides Lab. (CAPL), Agriculture Research Center, Giza, Egypt.

Enrichment and selection of pesticides degrading bacteria: Soil samples were transferred to the laboratory and were first enriched with access of chlorpyrifos and diazinon as organophosphorus pesticides for 10 days after thorough mixing to isolate potent bacterial isolates in organophosphorus pesticides biodegradation. Further enrichments were then carried out in which 1 g from previously enriched soils to inoculate 50 mL autoclaved minimal salt media (MSM)[19,20] containing 600 mg L^{-1} diazinon and 480 mg L^{-1} chlorpyrifos as a sole carbon source. Cultures were then incubated on an orbital shaker (Thermo fisher scientific, UK) at 30°C for 5 days at 150 rpm.

All cultures were allowed to settle for 2 h and 5 mL of each supernatant was used to inoculate 45 mL fresh MSM media containing the same ingredients for additional 7 days under the same conditions. Serial dilutions of cultures 10^{-4}-10^{-6} were plated on LB agar plates. Plates were incubated at 30°C for 18 h.

Degradation of chlorpyrifos and diazinon in minimal salt liquid media: The bacterial isolates were transferred to MSM media containing diazinon (600 mg L^{-1}) and chlorpyrifos (480 mg L^{-1}) as a sole carbon source. Each sample was prepared in triplicate. All cultures were incubated at 30°C and pH equal 7, 8 and 9 on an orbital shaker (Thermo fisher scientific, UK) at 150 rpm. At the same time non-inoculated media were also run in parallel to the other cultures as control samples were taken 0, 5, 10, 15 and 20 days after inoculation and the residual diazinon and chlorpyrifos were determined in the culture extract of the bacterial isolates and measured using gas chromatography (GC) analysis[3].

Extraction of genomic DNA from bacterial isolates: The isolated bacteria were cultured in conical flasks (Pyrex, USA) containing 20 mL LB medium by shaking in an orbital shaker (Thermo fisher scientific, UK) at 180 rpm for 18 h. The cultures were centrifuged at 13,000 rpm for 5 min at 4°C. The pellets were subjected to genomic DNA extraction using the (QIAamp DNA Mini Kit, QIAGEN, Germany). The extracted DNA was used as a template for PCR to amplify 16S rDNA and *Gro*El genes.

Molecular identification of bacterial isolates by PCR amplification and sequencing of 16S rDNA and *Gro*EL genes: Molecular tools such as 16S rDNA and *Gro*EL genes PCR amplification were used to identify the bacterial isolates[21,22]. Bacterial 16S rDNA was amplified by PCR using the universal primers; forward primer sequence (5'AGAGTTTGATCCTGGCTCAG3') and reverse primer sequence (5-CTACGGCTACCTTGTTACGA3-) thereby producing an amplicon of ~1500 bp. The *Gro*EL gene was amplified by PCR using the primers, forward primer sequence (5'GACGTCGCCGGTGACGGCACCACCAC3') and reverse primer sequence (5'CGACGGTCGCCGAAGCCCGGGGCCTT3'), thereby producing an amplicon of ~620 bp. In both reactions, amplification was carried out in 50 µL reactions by using a PCR master mix kit (Qiagen, Germany) according to the manufacturer's instructions using a GeneAmp PCR System 2400 Thermal cycler (Perkin-Elmer Norwalk, Connecticut, USA). The following program was used: 94°C for 3 min as initial denaturation step, followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 1 min for 16S rDNA gene amplification and 60°C for *Gro*EL gene amplification, extension at 72°C for 2 min, with a final extension at 72°C for 10 min. The PCR products were purified by QIAquick Gel Extraction Kit (Qiagen, Germany) following resolving by electrophoresis on 1% agarose gels and compared to a 1 kb DNA Ladder. Nucleotide sequence was determined using the same Primers with the dideoxy-chain termination method. The obtained sequences were analyzed for similarities to other known sequences found in the GenBank database using BLAST program of the NCBI database.

Identification of the degradation products by GC/MS: Bacterial isolate, GH10 was allowed to grow in the same media containing diazinon (600 mg L^{-1}) and chlorpyrifos (480 mg L^{-1}) separately as a sole carbon source for 10 days and incubated under the same previous conditions. Degradation products were extracted according to APHA[23] and identified by GC/MS.

PCR detection and amplification and of *oph* gene; the gene might be responsible for OPs degradation in the bacterial isolates: Sequences of *oph* deduced protein in all bacterial isolates were predicted based on the conserved domain database (CDD) of NCBI. The *oph* gene of putative organophosphorus hydrolase (oph) enzyme in bacterial isolate GH2NO8 was detected by analysis of genomic sequence of *Pseudomonas aeruginosa* isolate B10W, complete genome GenBank CP017969 and the prediction of ORFs. To amplify the *oph* gene excluding stop codon (NCBI Reference Sequence: WP_003114721.1; region (556862 to 557728), the forward primer GH2NO8-F (5-CAACAGCCAGAAACTCGACG⁻3) and the reverse primer GH2NO8-R (5⁻GGTGTCGTAGAGGCTGTAGC⁻3) were designed. The forward primer GH9OP-F (5 -CTGCAATACATGTTCATGCC-3') and the reverse primer GH9OP-R (5'-TCAGTAGTCCCAGA TGACCG-3') were used to amplify the *oph* gene in GH9OP bacterial strain based on the region (1841224 to 1842159) in the genome of *Achromobacter xylosoxidans* strain NH44784-1996 complete genome GenBank HE798385. In case of *oph* gene of bacterial isolate GH10, deduced protein sequence was predicted based on conserved domain database (CDD) of NCBI to amplify the *oph* gene, NCBI Reference Sequence: WP_007723364.1 region 1102101 to 1103057, analysis of complete genome of *Cronobacter muytjensii* ATCC 51329, GenBank: CP012268, forward primer GH10-F (5'-CTATTACCGCATGATGCTGGG-3') and reverse primer GH10-R (5'-CAGCGATACCCTTCTCCCTG-3') were designed. The PCR amplification of *oph* gene from the genomic DNA of bacterial isolates was conducted with GeneAmp PCR system 400 thermal cycler (PerkinElmer, Norwalk, Connecticut, USA). In this reaction amplification was carried out in a 50 µL reaction mixture by using a PCR master mix kit (Qiagen, Germany) according to the manufacturer's instructions. The following program was used: 94°C for 3 min as initial denaturation step, followed by 35 cycles of

denaturation at 94°C for 30 sec, annealing at 55°C for 1 min for bacterial isolate GH2NO8 *oph* gene, 50°C for 1 min for bacterial isolate GH9OP *oph* gene and 55°C for 1 min for bacterial isolate GH10 *oph* gene amplification, respectively and extension at 72°C for 1 min and a final extension step at 72°C for 10 min. PCR products were resolved by electrophoresis on 1% agarose gels and compared to a 100 bp DNA Ladder. Purified PCR products were sequenced by using the same primers and the obtained sequences were compared to other known sequences found in the database using the Blast program (http://www.ncbi.nlm.nih.gov/BLAST/).

RESULTS

Detection the most persistent pesticides residues in the Egyptian soil samples: As Table 1 shows that, chlorpyrifos was detected in all soil samples. The same situation was repeated in diazinon except, it was not detected in Sharqia. They recorded higher concentrations than that of other detected pesticides as Oxamyl and chlorpyrifos methyl. The highest concentration of Chlorpyrifos (0.09 mg kg^{-1} soil) was recorded in Kaliopia. However, the highest concentration of Diazinon (0.08 mg kg^{-1} soil) was recorded in Beheira.

Isolation of potent organophosphorus degrading bacterial isolates: Few bacterial isolates capable of degrading organophosphorus pesticides have been reported till date. Organophosphorus degrading bacteria were successfully isolated by using the enrichment technique, four different aerobic bacterial isolates capable of degrading chlorpyrifos of initial concentration (480 mg L^{-1}) and diazinon of initial concentration (600 mg L^{-1}) by the enrichment technique were developed from four soil samples collected from various agricultural fields. These four bacterial isolates labeled as, GH10, GH2NO8, GH9OP and GH4SNO/P have showed a good growth on minimal salt media containing diazinon and chlorpyrifos as the sole carbon source after 10 days (Fig. 1, 2).

Molecular identification of OPs degrading bacterial isolates by PCR amplification of the 16S rDNA and *Gro*El gene: The 16S rDNA gene universal primers amplified ~1550 bp and *Gro*El universal primers amplified ~620 bp for all bacterial isolates (Fig. 3, 4). Partial sequences were analyzed according to https://blast.ncbi.nlm.nih.gov, bacterial isolate GH10 was identified as *Cronobacter muytjensii* strain, GH10, bacterial isolate GH2NO8 was identified as *Pseudomonas aeruginosa* strain GH2NO8, bacterial isolate GH9OP was identified as *Achromobacter xylosoxidans* strain GH9OP and GH4SNO/P bacterial isolate was identified as *Pseudomonas putida* strain GH4SNO/P. The 16S rDNA and *Gro*El genes partial sequences of bacterial isolates were deposited in the GenBank database with accession numbers shown in Table 2.

Table 1: Concentrations of pesticides residues detected in the soil samples

Soil sample locations	Detected pesticides	Concentration (mg kg^{-1} soil)
Giza	Chlorpyrifos	0.01
	Oxamyl	0.002
	Aldicarb	0.004
	Diazinon	0.02
Sharqia	Oxamyl	0.004
	Chlorpyrifos	0.05
Beheira	Chlorpyrifos methyl	0.07
	Oxamyl	0.007
	Diazinon	0.08
Kaliopia	Oxamyl	0.004
	Chlorpyrifos	0.09
	Diazinon	0.008
Monofia	pirimiphos methyl	0.006
	Diazinon	0.04
	Chlorpyrifos	0.07

Fig. 1: Growth of bacterial isolates on liquid minimal salt media containing chlorpyrifos (480 mg L^{-1}) after 10 days incubation. GH9OP, GH2NO8, GH10 and GH4SNO/P were the bacterial isolates, Control: Liquid MSM with chlorpyrifos (480 mg L^{-1}) without bacterial inoculum

Fig. 2: Growth of bacterial isolates on liquid minimal salt media containing diazinon (600 mg L^{-1}) after 10 days incubation. GH9OP, GH2NO8, GH10 and GH4SNO/P were the bacterial isolates; Control: Liquid MSM with diazinon (600 mg L^{-1}) but no bacterial inoculum

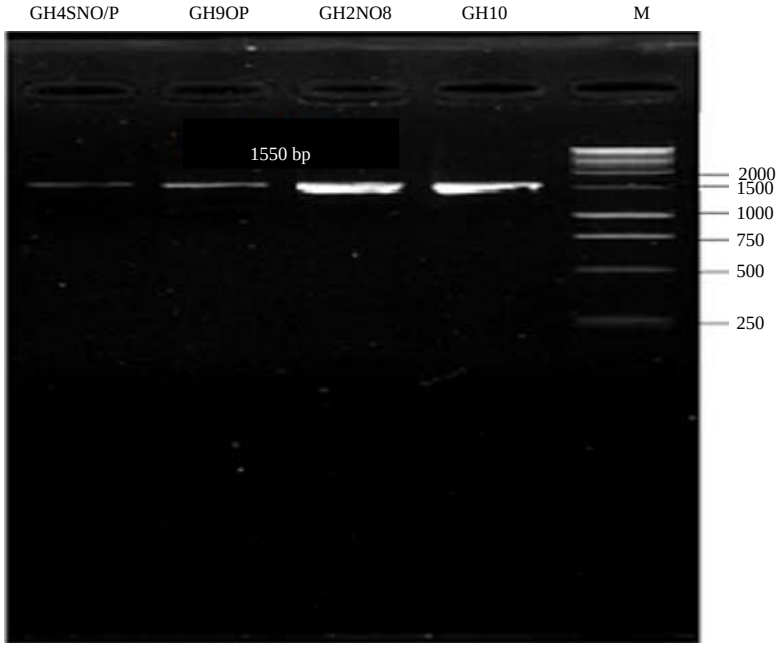

Fig. 3: Agarose gel electrophoresis for PCR product of 16s rDNA gene in bacterial isolates, (GH10, GH2NO8, GHOP and GH4NO/P); M: 1 kb DNA ladder

Table 2: Accession numbers of 16s rDNA and *Gro*El genes of bacterial isolate

Bacterial isolates codes	Molecular identification	Accession number of 16s rDNA gene	Accession number of *Gro*El gene
GH10	*Cronobacter muytjensii* strain GH10	KY945346	KY945344
GH2NO8	*Pseudomonas aeruginosa* strain GH2NO8	KY945349	LC224324
GH9OP	*Achromobacter xylosoxidans* strain GH9OP	KY945347	KY945345
GH4SNO/P	*Pseudomonas putida* strain GH4SNO/P	KY945348	LC224325

Degradation of diazinon and chlorpyrifos in liquid MSM by bacterial isolates: *Cronobacter muytjensii* strain GH10 was able to degrade 92.59% of diazinon as compared to 16.99% in control and 93.43% of chlorpyrifos as compared to 4.28% in control (Fig. 5) after 20 days of incubation. This search has been the first study that manipulates *Cronobacter muytjensii* in pesticides biodegradation where this bacterial isolate showed a remarkable organophosphorus pesticides biodegradation.

There was a considerable removal of diazinon, *Pseudomonas aeruginosa* strain GH2NO8, *Achromobacter xylosoxidans* strain GH9OP and *Pseudomonas putida* strain

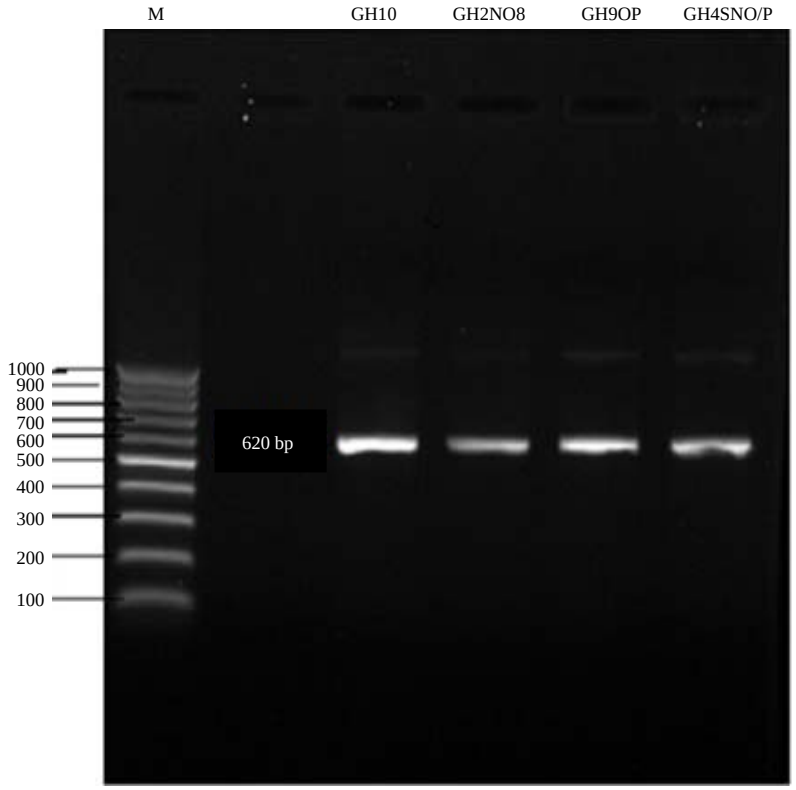

Fig. 4: Agarose gel electrophoresis for PCR product of *Gro*EL gene bacterial isolates: GH10, GH2NO8, GHOP and GH4NO/P; M: 100 bp DNA ladder

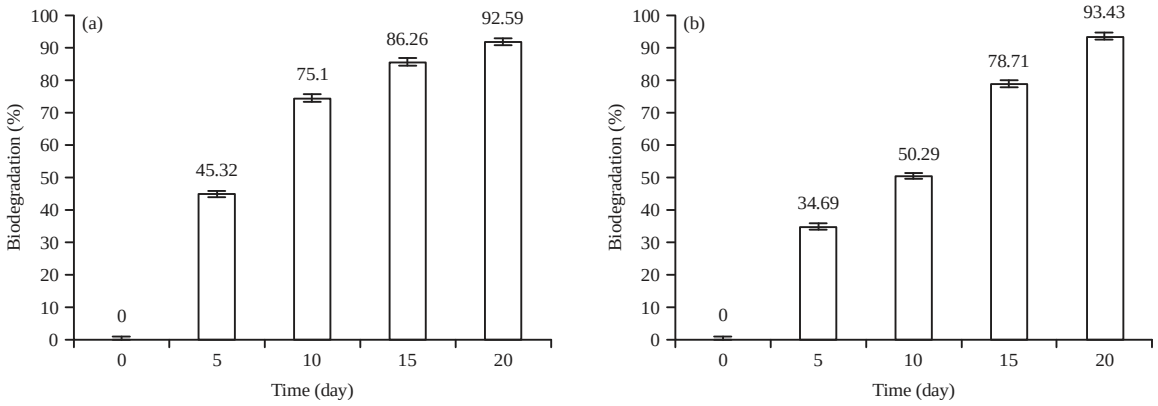

Fig. 5(a-b): (a) Biodegradation (%) of diazinon (600mg L^{-1}) and (b) Biodegradation of chlorpyrifos (480 mg L^{-1}) by *Cronobacter muytjensii* strain GH10

GH4SNO/P were able to degrade 91.82, 97.75 and 90.78%, respectively as compared to 16.99% in control. With respect to chlorpyrifos biodegradation, these bacterial isolates exhibited the ability to remove 78.51, 93.18 and 95.36% as compared to 4.28% in control after 20 days of incubation (Fig. 6-9). In diazinon biodegradation, *Achromobacter xylosoxidans* strain GH9OP showed the highest degradation, but in Case of chlorpyrifos biodegradation, *Pseudomonas putida* strain GH4SNO/P recorded the highest degradation.

Identification of the degradation products of diazinon and chlorpyrifos by GC/MS: In this study, it is the first time to manipulate *Cronobacter muytjensii* in pesticides biodegradation so that *Cronobacter muytjensii* strain GH10

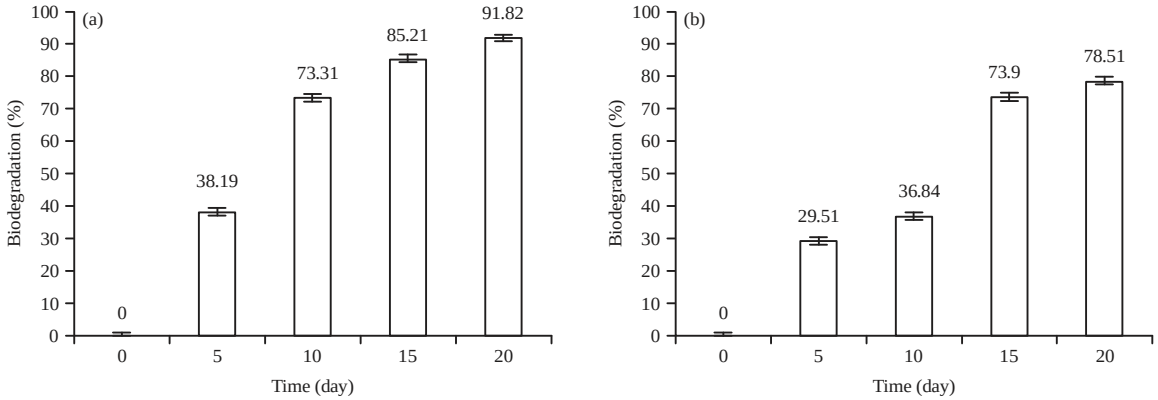

Fig. 6(a-b): (a) Biodegradation (%) of diazinon (600mg L^{-1}) and (b) Biodegradation of chlorpyrifos (480 mg L^{-1}) by *Pseudomonas aeruginosa* strain GH2NO8

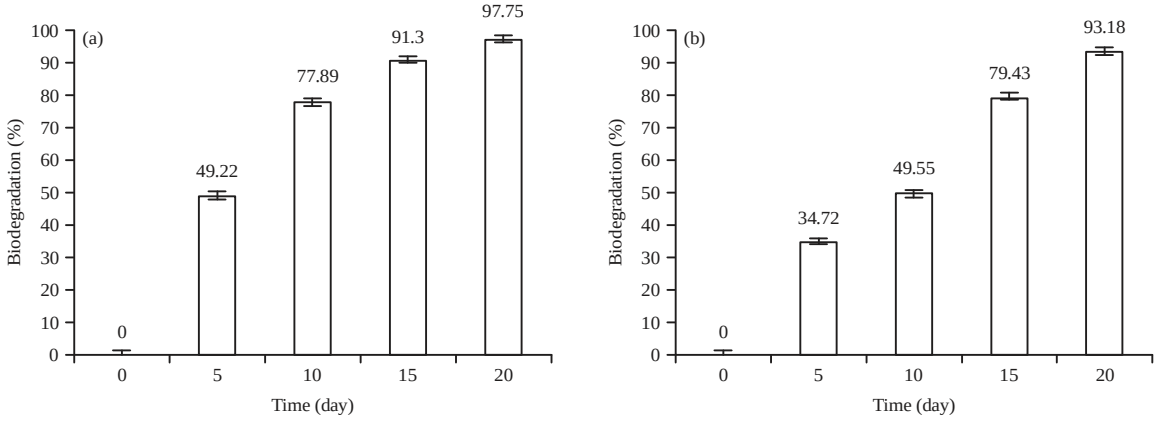

Fig. 7(a-b): (a) Biodegradation (%) of diazinon (600 mg L^{-1}) and (b) Biodegradation of chlorpyrifos (480 mg L^{-1}) by *Achromobacter xylosoxidans* strain GH9OP

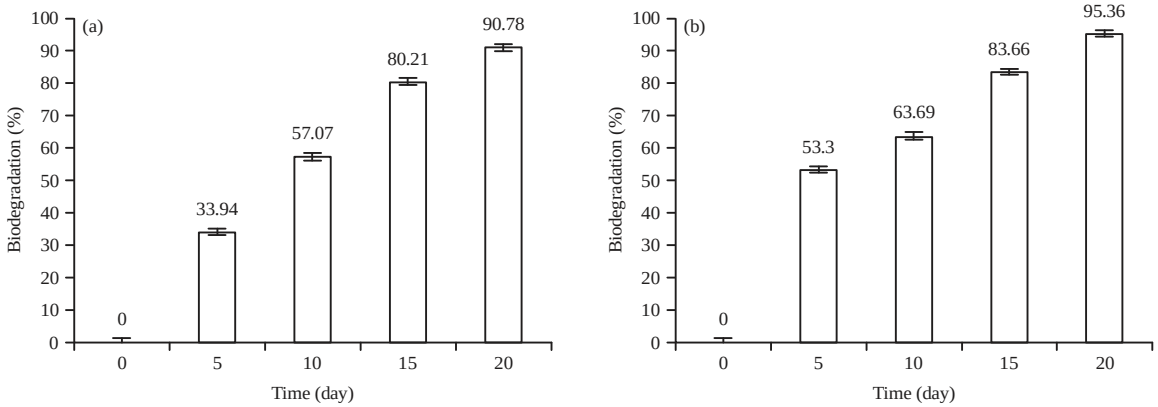

Fig. 8(a-b): (a) Biodegradation (%) of diazinon (600mg L^{-1}) and (b) Biodegradation of chlorpyrifos (480 mg L^{-1}) by *Pseudomonas putida* strain GH4SNO/P

can be used as a model strain to detect diazinon and chlorpyrifos metabolites. Diazinon insecticide is a phosphorothionate moiety which belongs to the main chemical group of organophosphorus[24]. 2-Iso-4-methyl-6-hydroxypyrimidine (IMHP) with molecular formula of $C_8H_{12}N_2O$ and molecular weight of 152.1 kDa was detected as the main degradation product of diazinon at m/z 137 , retention time (Rt = 20.3200 min) (Fig. 10) these peaks disappeared

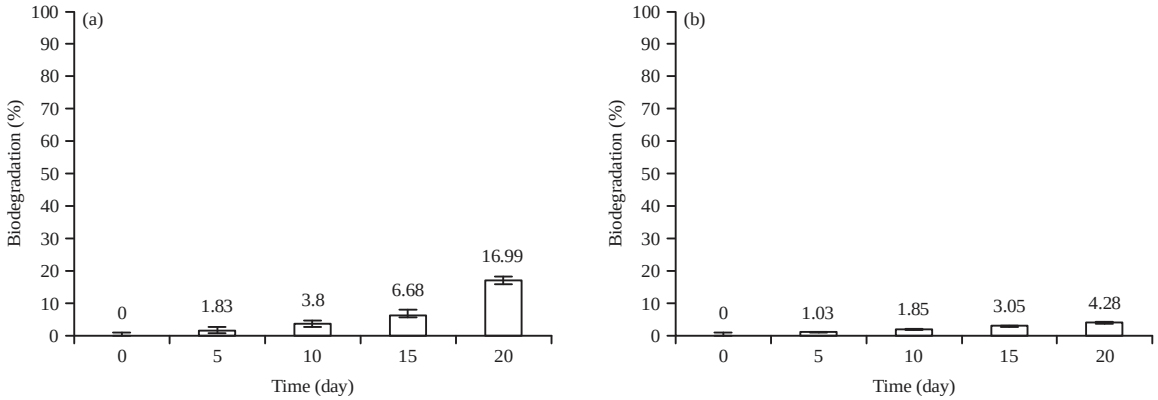

Fig. 9(a-b): (a) Biodegradation (%) of diazinon (600 mg L^{-1}) and (b) Biodegradation of chlorpyrifos (480 mg L^{-1}) in control media

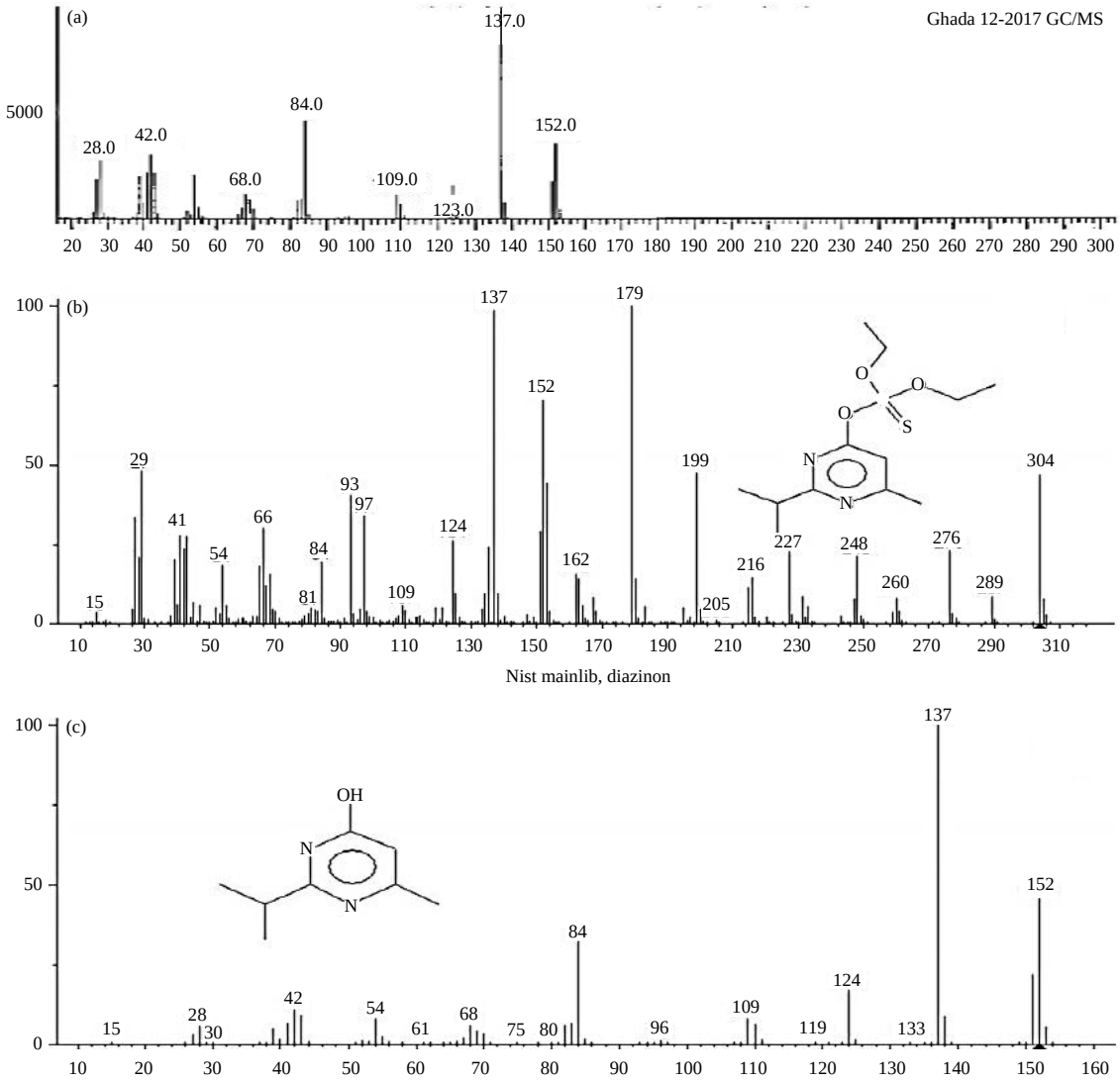

Fig. 10(a-b): Mass spectra of 2-Iso-4-methyl-6- hydroxypyrimidine (IMHP) produced from diazinon degradation by (a) *Cronobacter muytjensii* strain GH10; Ghada: Sample, (b) Authentic standard diazinon and (c) Authentic standard IMHP from the National Institute of Standards and Technology (NIST, USA) library database

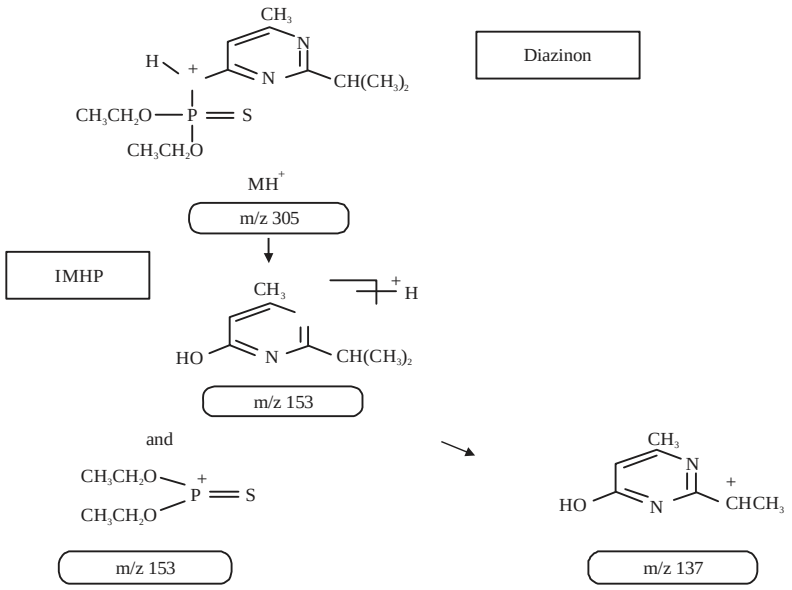

Fig. 11: Proposed pathway for the diazinon degradation by *Cronobacter muytjensii* strain GH10

concomitantly with formation of other new peaks with a retention time of around 41.38. Subsequently, the hydrolysis product, IMHP was further transformed by ring breakage, resulting in its detoxification. The degradation pathway for diazinon by *Cronobacter muytjensii* strain GH10 was proposed (Fig. 11). With respect to chlorpyrifos biodegradation, the metabolic products of chlorpyrifos were based on the characteristic fragment ion peaks and molecular ion m/z 197. The new peak was identified as 3,5,6-trichloro-2-pyridinol (TCP) as the main metabolite of chlorpyrifos at retention times (RT) of 30.03 min. This peak disappeared concomitantly with formation of other new peaks with a retention time of around 24.1 min (Fig. 12). Each peak was identified on the basis of its mass spectra and the NIST library identification program. The degradation pathway for chlorpyrifos was proposed (Fig. 13). Subsequently, the hydrolysis product, TCP was further transformed by ring breakage, resulting in its detoxification.

PCR amplification and partial sequence analysis of *oph* gene in the bacterial isolates: The primers GH10-F and GH10-R successfully amplified ~820 bp, ~810 bp pcr amplicon was produced by primers GH2NO8-F and GH2NO8-R and primers GH9OP-F and GH9OP-R amplified ~740 bp (Fig. 14) *oph* genes are sequenced and subjected to a analysis on (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), all three *oph* genes sequences are members of the MBL-fold metallo hydrolase superfamily and have beta lactamase fold which are mainly hydrolytic enzymes involved in organophosphates hydrolysis[25]. Deduced amino acids sequences of *oph* gene in bacterial isolates were aligned using CLUSTAL multiple sequence alignment using MUSCLE 3.8. The translated amino acid sequence of *oph* gene from *Cronobacter muytjensii* strain has 84% similarity with *mpd Cronobacter sakazakii* ATCC BAA-894. Accession No. CP000783, in case of *Achromobacter xylosoxidans* strain GH9OP, it has 99% similarity with *opd* gene of *Achromobacter xylosoxidans* NH44784-1996 Accession no. HE798385 (Fig. 15-19) and *Pseudomonas aeruginosa* strain GH2NO8 *oph* gene has 100% similarity with that of *Pseudomonas aeruginosa* isolate B10W Accession No. CP017969 (Fig. 20). There was no similarity between *oph* gene DNA sequences in bacterial isolates manipulated in this study. Based on (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), the conserved domains of these isolated genes are that present in *Pseudomonas pseudoalcali genes* OPHC2 which is a thermostable organophosphorus hydrolase, it hydrolyzes various phosphotriester, esters and a lactone. This subgroup also includes *Pseudomonas oleovorans* PoOPH which exhibits high lactonase and esterase activities and latent PTE activity. The *oph* gene in *Cronobacter muytjensii* strain GH10, *Achromobacter xylosoxidans* GH9OP and *Pseudomonas aeruginosa* strain GH2NO8 are firstly isolated by us from Egyptian soils, these sequences are deposited in GenBank database under the accession numbers MF443872, MF443871 and MF443870, respectively.

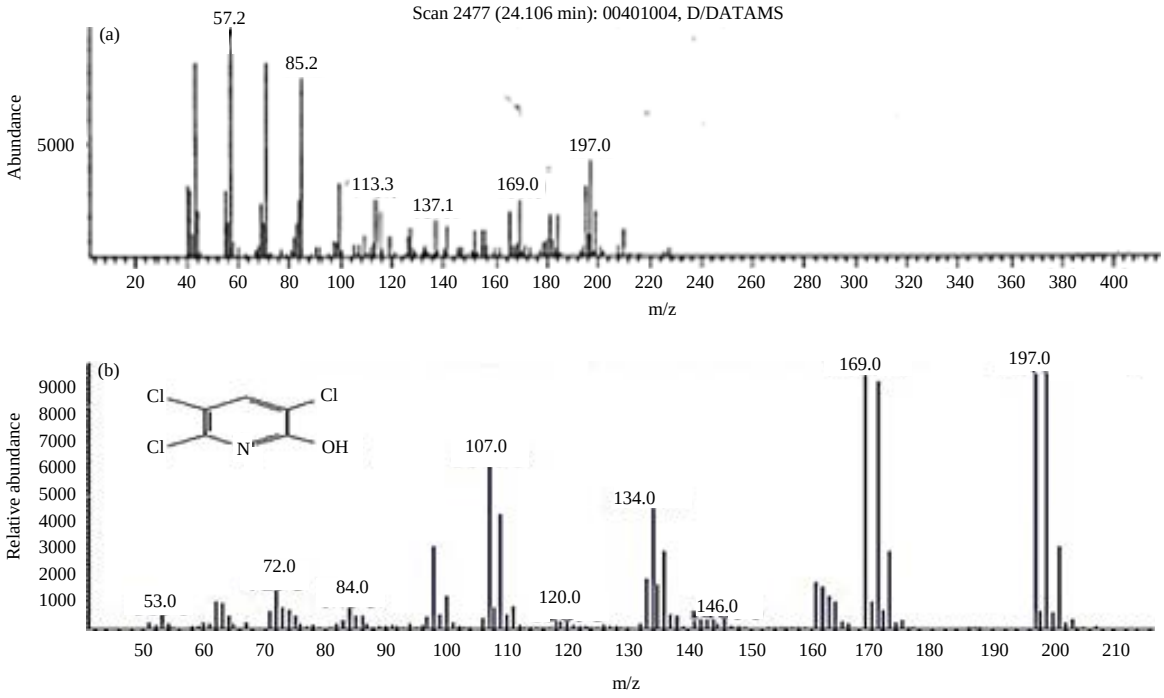

Fig. 12: Mass spectra of 3,5,6-trichloro-2-pyridinol (TCP) produced from chlorpyrifos degradation by *Cronobacter muytjensii* strain GH10. M: Sample; N: Authentic standard TCP from the National Institute of Standards and Technology (NIST, USA) library database

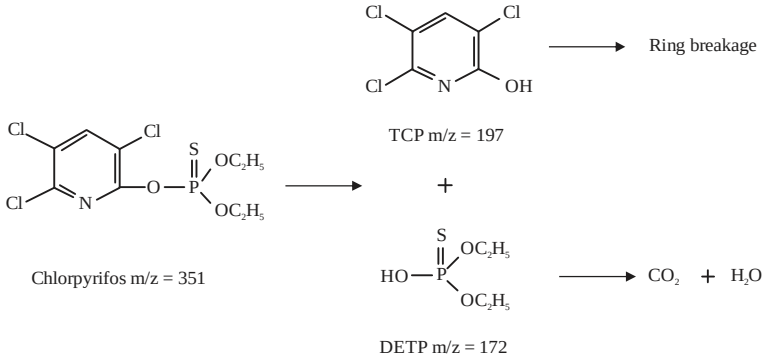

Fig. 13: Proposed pathway for the chlorpyrifos degradation by *Cronobacter muytjensii* strain GH10

DISCUSSION

Chlorpyrifos and diazinon are very popular pesticides that are used extensively for the purpose of pest control in vegetables and cotton fields. Presence of these pesticides were detected by Metwally *et al.*[26], it discovered that organophosphorus pesticides, diazinon and chlorpyrifos were the most persistent pesticides in different Egyptian soils. These pesticides are very toxic for mammals and can lead to contamination in environmental niches Bioremediation is a process that utilizes micro-organisms for pesticides degradation For this purpose, OPs degrading bacterial strains were isolated. Four bacterial strains were isolated from pesticides contaminated agriculture soils. They showed high degradation potential in diazinon and chlorpyrifos at high concentration. In a few previously published reports, bacterial strain *Achromobacter xylosoxidans* (JCp4) and *Ochrobacterum* sp. (FCp1) were able to degrade chlorpyrifos in sterilized and non-sterilized soils and exhibited the ability to degrade 93-100% of the input concentration 200 mg L^{-1} within[1] 42 days. In this study, *Achromobacter xylosoxidans* strain GH9OP is a promising candidate for raising the

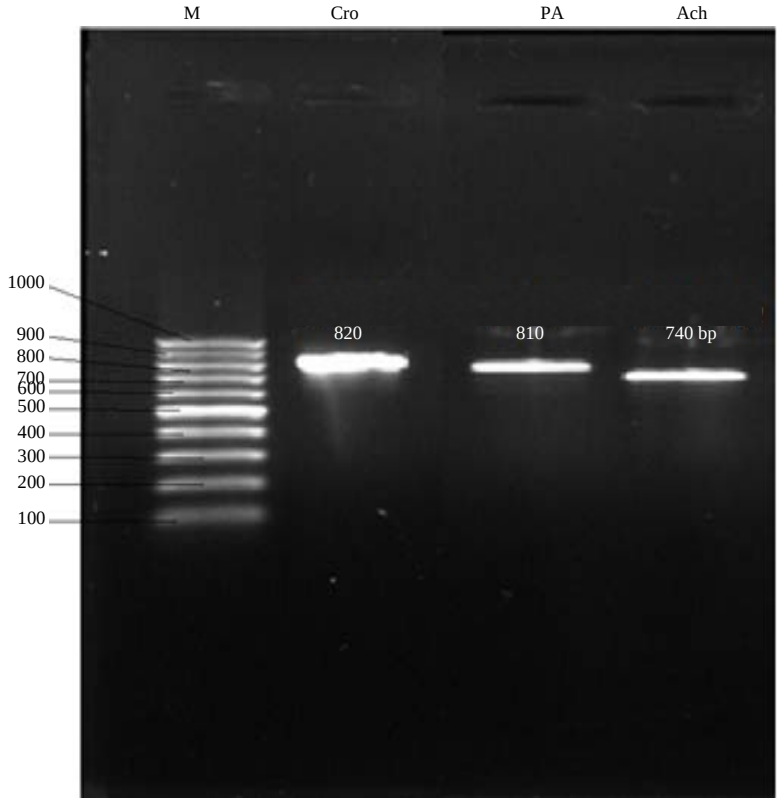

Fig. 14: Agarose gel electrophoresis for PCR product of *oph* gene
M: DNA ladder, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000) Cro: *Cronobacter muytjensii*. PA: *Pseudomonas aeruginosa*. Ach: *Achromobacter xylosoxidans*

TAVSDGTVTIPTDKLLTRITPQALNARLADDALTSQVETSINTYVINTGDRLILVDT
GAGPLMGDAGGHLPENLRAAGIDPAQIDTVLLTHVHGDHSGGVQRDGKLAFP
NATVRVDQRDVDLWLNPARKNEVEESQRHTFAESERSLRPVIDAGKLGTFRAPT
QIMPGIEALPAPGHTPGSVIYKVSRGGETLLLWGDIIHVKAVQMPQPQVAIHFDV
NQDGAVATREKTLKM

Fig. 15: Deduced amino acid sequence of the new *oph* gene from *Cronobacter muytjensii* strain GH10

productivity of crops in pesticides contaminated soils. Many bacterial strains are involved in organophosphorus pesticides degradation, among these bacteria, *Enterobacter* sp.[17], *pleismonas* sp.[27], *Agrobacterium radiobacter*[22] and *Streptomyces* sp.[28]. *Pseudomonas aeruginosa* strain GH2NO8 and *Pseudomonas putida* strain GH4SNO/P recoded high chlorpyrifos and diazinon degradation activity, these results are agreed with that obtained by Khani and Kafilzadeh[29], where he isolated *Pseudomonas aeruginosa* and *Flavobacterium* sp. that were able to reduce the level of diazinon at level of $p \leq 0.05$. Khalid *et al.*[30] isolated *Pseudomonas putida* CP-1, this bacterium was able to hydrolyze the phosphotriester bonds in chlorpyrifos. Moreover, *Pseudomonas putida* MAS-1 able to degrade chlorpyrifos was isolated by Ajaz *et al.*[31]. Bacterial strains manipulated in this study were distinguished from other bacterial strains manipulated in other reports, they were tolerant to high OPs concentrations which were toxic for the second. Microbial biodegradation of chlorpyrifos and diazinon might be due to chemical resemblance between them. A novel bacterial strain, *Cronobacter muytjensii* GH10 was firstly manipulated in OPs biodegradation. It was used as a model strain to detect diazinon and chlorpyrifos metabolites. It was able to degrade both pesticides to non-polar compounds and OPs detoxification. It is worthy mentioned that 2-isopropyl-4-methyl-6 hydroxypyrimidine as the degradation product of diazinon previously is less toxic than diazinon[24] , for this reason, *Cronobacter muytjensii* GH10 is considered a model strain in diazinon detoxification. Moreover, this strain degraded chlorpyrifos to nonpolar products, these results indicated that the added chlorpyrifos (480 mg L^{-1}) was degraded without any accumulative products after 20 days of incubation. This feature is rarely reported in other chlorpyrifos-degrading microorganisms where in most cases reported to date, the individual isolate was able to transform

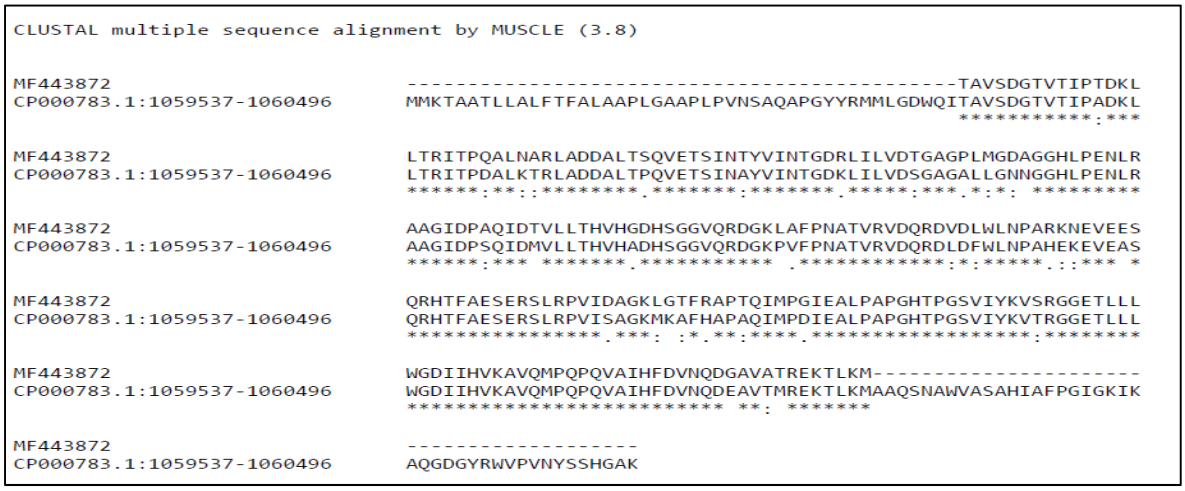

Fig. 16: Pairwise alignment of deduced amino acids sequences of *oph* gene from *Cronobacter muytjensii* strain GH10 accession No. MF443872 and (*mpd*) gene from *Cronobacter sakazakii* ATCC BAA-894. Accession No. CP000783.1:1059537-1060496 encodes methyl parathion hydrolase as organophosphorus hydrolase enzyme

LNVMVARSGDQTILIDAGLGGQFPGFPRAGQLPQRLEDAGIALESVTDVIITHM
HMDHVGGLLVDGVKERLRPDVRIHVSATEVAFWTSPDFSHTVMPKPVPAVLRST
AASFYNEYRDRLRIFQDRHEVAPGVVVRITGGHTPGHSVVDLIGGDERLTFAGD
AIFPVGFDHPDWHNGFEHDPEESARVRLRLFQELAQNRGLLVAAHLPFPSVGRV
AIDGDAFRWVPVI

Fig. 17: Deduced amino acid sequence of the new *oph* gene from *Achromobacter xylosoxidans* strain GH9OP

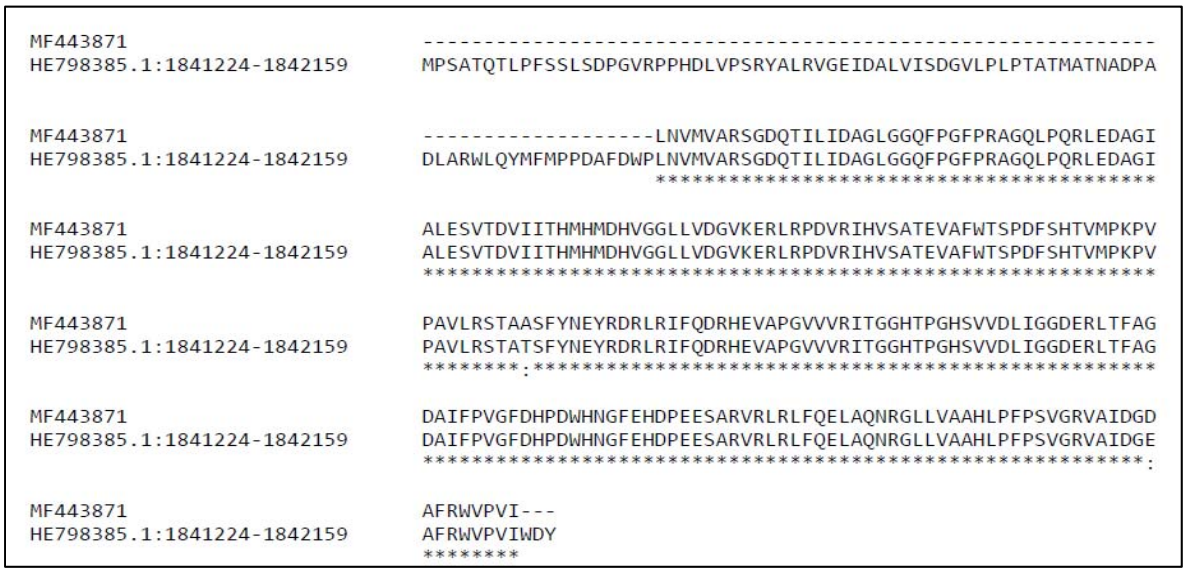

Fig. 18: Pairwise alignment of deduced amino acids sequences of *oph* gene from *Achromobacter xylosoxidans* strain GH9OP accession No. MF443871 and organophosphorus hydrolase enzyme (*oph*) gene from *Achromobacter xylosoxidans* NH44784-1996 Accession No. HE798385.1:1841224-1842159

FGNAPRALWSRWMQPDAENRIDLGCRALLVRDGERNVLVETGIGAFFPPALRQR
YGVQEERHVLLDSLAAVGLDDADIDVVLLTHLHFDHAGGLLAAWEEGQPARLL
FPNAHFVSGRRHWQRARQPHPRDRASFVPELLDLLQASGRLELLDDGERSAHLG
EGWRFHFSEGHTPGQMLPEIAMPDGPVVFSGDLIPGAPWVHLPLTMGYDRFPEG
LIEEKERLLDSLIARNGRLVFTHDPCVAMGRVRRDEQERYSLYDTLER

Fig. 19: Deduced amino acid sequence of the new *oph* gene from *Pseudomonas aeruginosa* strain GH2NO8

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CLUSTAL multiple sequence alignment by MUSCLE (3.8)


MF443870                        ----------FGNAPRALWSRWMQPDAENRIDLGCRALLVRDGERNVLVET
CP017969.1:c557728-556862       MRTLTTLLGNSQKLDGGAMFGNAPRALWSRWMQPDAENRIDLGCRALLVRDGERNVLVET
                                          ****************************************

MF443870                        GIGAFFPPALRQRYGVQEERHVLLDSLAAVGLDDADIDVVLLTHLHFDHAGGLLAAWEEG
CP017969.1:c557728-556862       GIGAFFPPALRQRYGVQEERHVLLDSLAAVGLDDADIDVVLLTHLHFDHAGGLLAAWEEG
                                ************************************************************

MF443870                        QPARLLFPNAHFVSGRRHWQRARQPHPRDRASFVPELLDLLQASGRLELLDDGERSAHLG
CP017969.1:c557728-556862       QPARLLFPNAHFVSGRRHWQRARQPHPRDRASFVPELLDLLQASGRLELLDDGERSAHLG
                                ************************************************************

MF443870                        EGWRFHFSEGHTPGQMLPEIAMPDGPVVFSGDLIPGAPWVHLPLTMGYDRFPEGLIEEKE
CP017969.1:c557728-556862       EGWRFHFSEGHTPGQMLPEIAMPDGPVVFSGDLIPGAPWVHLPLTMGYDRFPEGLIEEKE
                                ************************************************************

MF443870                        RLLDSLIARNGRLVFTHDPCVAMGRVRRDEQERYSLYDTLER------
CP017969.1:c557728-556862       RLLDSLIARNGRLVFTHDPCVAMGRVRRDEQERYSLYDTLERVENLEA
                                *****************************************
```

Fig. 20: Pairwise alignment of deduced amino acids sequences of *oph* gene from *Pseudomonas aeruginosa* strain GH2NO8 accession No. MF443870 and organophosphorus hydrolase enzyme (*oph*) gene from *Pseudomonas aeruginosa* isolate B10W Accession No. CP017969.1:c557728-556862

chlorpyrifos by hydrolysis of ester linkage and gave TCP, which in turn accumulated in the batch cultures or soils moreover, enhanced degradation could not occur due to its antimicrobial properties[32,33]. The TCP has estrogenic activity and has recently been listed as potential endocrine disrupting chemicals by the Environmental Protection Agency (EPA) of the USA[34]. Compared with the importance of TCP degradation issue, studies concerning its degradation and fate in the environment are very limited. Chen *et al.*[35] isolated new fungal strain Hu-01 identified as *Cladosporium cladosporioides* with high chlorpyrifos-degradation activity and utilized 50 mg L^{-1} of chlorpyrifos as the sole carbon of source at acidic pH 6.5.This study contrasted with his previous findings, where chlorpyrifos and diazinon degradation by bacterial isolates in this study was perfect in basic pH that is already present in most Egyptian agriculture soils. Another important feature which is worthy mentioned is that bacterial isolates under study engaged in efficient degradation of diazinon and chlorpyrifos at high concentration in contrast to other reports that stated the toxic effects of OPs on diverse microorganisms[36,37]. High chlorpyrifos and diazinon tolerance and degradation capability of the bacterial isolate makes these strains suitable for decontamination and bioremediation of contaminated sites.

Bacterial strains have organophosphorus degrading genes that encode organophosphorus hydrolase (OPH) enzymes to degrade OPs. The DNA sequences of oph gene in bacterial isolates belonged to metallo beta lactamase super family which is mainly involved in OPs biodegradation. They have no similarity among them, although they perform the same function. This phenomenon was emphasized by Salman *et al.*[5], reported that a total of 60 different enzymes involved in organophosphate degradation are divided into 8 subgroups based on the type of organism and no significant homology is observed overall and poorly aligned regions at the ends of proteins can be easily seen, moreover, the active sites in all these enzymes do not possess conserved amino acid sequences and consequently they give rise to a different active site structure.

CONCLUSION

This study demonstrated that diazinon and chlorpyrifos as organophosphorus pesticides were the most persistent pesticides residues in Egyptian agriculture soils, four potent bacterial isolates in Ops biodegradation were molecularly identified as *Cronobacter muytjensii* strain GH10, *Pseudomonas aeruginosa* strain GH2NO8, *Achromobacter*

xylosoxidans strain GH9OP and *Pseudomonas putida strain* GH4SNO/P by 16s rDNA and *Gro*El genes PCR amplification. Gene responsible for OPs biodegradation, *oph* gene in *Cronobacter muytjensii* strain GH10, *Pseudomonas aeruginosa* strain GH2NO8 and *Achromobacter xylosoxidans* strain GH9OP was detected for the first time in this study. The metabolites produced due to diazinon and chlorpyrifos biodegradation by *Cronobacter muytjensii* strain GH10 as a new strain has proved that this strain is a good tool in bioremediation process in contaminated soil and water.

SIGNIFICANCE STATEMENT

This study confirmed that organophosphorus pesticides are considered among the most persistent pesticides in Egyptian agriculture soils. Bacterial isolates *Cronobacter muytjensii* strain GH10, *Pseudomonas aeruginosa* strain GH2NO8, *Achromobacter xylosoxidans* strainGH9OP and *Pseudomonas putida* strain GH4SNO/P are good candidates for bioremediation process.

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