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Cloning of the Organophosphorus Hydrolase (*oph)* Gene and Enhancement of Chlorpyrifos Degradation in the *Achromobacter xylosoxidans* Strain GH9OP via Mutation Induction

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Abstract

In this study, the *Achromobacter xylosoxidans* strain GH9OP showed an effective activity in chlorpyrifos degradation. This bacterial strain was able to degrade 34.72 % of chlorpyrifos compared to 1.03 % in the control media after five days. The *oph* gene of the *Achromobacter xylosoxidans* strain GH9OP, which encodes a protein involved in chlorpyrifos hydrolysis, was cloned and sequenced. It is a member of the MBL-fold metallo hydrolase superfamily, and has a beta lactamase fold. Moreover, it has 99 % similarity with that of *Achromobacter xylosoxidans* NH44784-1996. The DNA sequence of the open reading frame of the *oph* gene was deposited in Genbank database under accession number MH018244. AchM15 and AchMS1 were the best mutants in the chlorpyrifos biodegradation after the ethylmethane sulphonate (EMS) mutation induction for the *Achromobacter xylosoxidans* strain GH9OP resulting from first-step and second- step EMS mutation induction, respectively. AchM15 was able to degrade 63 % of chlorpyrifos compared to 34 % in wild type after five days. However AchMS1 exhibited the ability to degrade 82.03 % of chlorpyrifos compared with 65 % by AchM15 after five days. 3, 5, 6- trichloro-2-pyridinol (TCP) as a metabolite of the chlorpyrifos biodegradation, which was further metabolized to unknown polar metabolites, was detected using GC/MS after ten days in the case of AchMS1.

Keyword: *Achromobacter xylosoxidans*, Chlorpyrifos, Biodegradation, *oph*, Gene cloning, Ethylmethane sulphonate mutation.

1. Introduction

From the most important factors contributing to the massive increase in food production worldwide is the use of pesticides in agricultural systems. Moreover, applications of pesticides have been an environmental concern for the past several decades (Wang *et al.,* 2012) and their widespread use has caused environmental pollution (Lew *et al.,* 2013; Liu *et al.,* 2013). Organophosphate (OPs) compounds are highly potent neurotoxins that are commonly used as pesticides. These compounds inhibit acetylcholine esterase and disrupt the normal function of the central nervous system followed by severe muscle paralysis and death (Carvalho *et al.,* 2003). OPs are much more toxic to vertebrates compared to other of insecticide classes (Malhat and Nasr, 2011). Chlorpyrifos (O,O-diethyl O-(3,5,6-trichloro-2-pyridyl phosphorothioate), for instance, has been extensively applied in the household and for agricultural pest control. It is a non-systemic insecticide and acaricide with contact, ingestion, and inhalation (Abraham *et al.,* 2016; Thabit and El-Naggar, 2013).

A high concentration of chlorpyrifos was determined in Egyptian soils (Metwally, 2014). There are several methods available for the detoxification of these OPs including chemical treatment, incineration, volatilization, and photodecomposition, but most of them are not applicable for the complete removal of pesticide contamination at high concentrations because they are not environmental friendly, inefficient, and expensive. Microbial biodegradation is a reliable and costly-effective technology used for removing organophosphates (OPs) from surface water and soils (Chishti *et al*., 2013; Abraham and Silambarasan, 2016). Several bacterial isolates have been reported to be potent in the OPs biodegradation such as *Flavobacterium* sp. (Sethunathan and Yoshida, 1973), *Arthrobacter* sp. (Mallick *et al.,* 1999) and *Pseudomonas diminuta* (Serdar *et al.,* 1982). Chlorpyrifos biodegradation by soil bacteria, comprising seven different isolates of *Pseudomonas*, *Bacillus* and *Agrobacterium,* has been investigated (Maya *et al.,* 2011).

Mutagenesis through classical genetic approaches involves the use of random mutations to improve the desired metabolites' yields and the bacterial enzymes' production, in particular, bacterial wild strains (Chen *et al.,* 2008). The advantages of random mutagenesis using

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chemical mutagens such as ethyl methyl sulphonate (EMS) are more overwhelming due to their simplicity and low cost procedures compared to DNA recombinant (Munazzah *et al.*, 2012; Rowland, 1984).

Organophosphorus hydrolase (OPH) enzyme are encoded by *oph* gene, *mpd* gene and/or *opd* gene in different unrelated bacterial strains. These genes have no a DNA- sequence similarity (Bigley and Raushel, 2013).

The objectives of this study are to identify and characterize the *oph* gene responsible for chlorpyrifos biodegradation in the *Achromobacter xylosoxidans* strain GH9OP. Improving the ability of this strain for chlorpyrifos biodegradation by chemical mutation induction using ethylmethane sulphonate (EMS) is the second goal of this research.

2. Materials and Methods

2.1. Reagents and Chemicals

Analytical grade chlorpyrifos (48 %) emulsifiable concentrate was purchased from (Sinochem Agro.Co.Ltd, China). All reagents and solvents used in the present study were of the analytical grade. Chlorpyrifos was used as an organophosphorus insecticide model due to its high residues in Egyptian soils (Metwally, 2014).

2.2. Evaluation the Chlorpyrifos Biodegradation by Achromobacter xylosoxidans Strain GH9OP

The *Achromobacter xylosoxidans* strain GH9OP isolated and identified in microbial genetic department, national research Centre, was friendly taken and inoculated in broth minimal salt media (Fang *et al.*, 2008) containing chlorpyrifos with a concentration of 480 mg/L as a sole carbon source and was incubated on an orbital shaker (Thermoscientific, UK) at 30 ^{o}C for five, ten, and fifteen days at 150 rpm, and samples were done in triplet (Singh and Walker, 2006). At the same time, non-inoculated media were also run in parallel to the other cultures as control. The extraction of chlorpyrifos residues was done as follow: a known volume of a mineral salt liquid media (MSL), 100 mL, was transferred into a 500 mL separatory funnel, and was partitioned successively three times with 50 mL of dichloromethane each and 40 mL of a sodium chloride solution (20 %). The combined extracts were filtered through a pad of cotton and anhydrous sodium sulfate, and were then evaporated at 30 ^{o}C to dryness using a rotary evaporator at 30^{o}C. After that, the residue was quantitatively transferred to a standard glass stopper test tube with ethyl acetate, and the solvent was evaporated to dryness (Metwally, 2014). The Chlorpyrifos residue and its metabolites were determined using Gas Chromatography analysis in the Central Agriculture Pesticides Lab (CAPL), Agriculture Research Center, Giza, Egypt. At the same time, 1 mL of the bacterial culture was used to make serial dilutions 10^{-4} to 10^{-6} and was plated on Luria-Bertani (LB) agar plate. The plate was incubated at 30 ^{o}C for eighteen hours, and a single colony was subjected to DNA extraction and EMS mutation induction.

2.3. Data Calculation

$$\text{Degradation (\%)} = \left(\frac{\text{Residual amount in blank control} - \text{Residual amount in sample}}{\text{Residual amount in blank control}}\right) \times 100$$

2.4. Extraction of Genomic DNA from the Achromobacter xylosoxidans Strain GH9OP

A Single colony was cultured in a conical flask (Pyrex, USA) containing 20 mL of an LB medium by shaking in an orbital shaker (Thermo fisher scientific, UK) at 150 rpm for eighteen hours. The culture was centrifuged at 13,000 rpm for five minutes at 4^{o}C. The pellet was 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 the *oph* gene.

2.5. Cloning and Sequencing of the oph Gene: The Gene That Might Be Responsible for Chlorpyrifos Degradation in the Achromobacter xylosoxidans Strain GH9OP

Sequence of the *oph* gene in *Achromobacter xylosoxidans* GH9OP was predicted based on search results from the conserved domain database (CDD) of NCBI, The *oph* gene of putative organophosphorus hydrolase (oph) enzyme was detected by the analysis of the complete genomic sequence of the *Achromobacter xylosoxidans* strain NH44784-1996 genbank HE798385 region, 1841224 to 1842159. PCR amplification of the *oph* gene by the sense primer F-oph 5´GGGGATCCATGCCATCCGCAACCCAAACCC´3 and antisense primer R-oph 5´GGAAGCTTTCAGTAGTCCCATATGACCGGC´3 using the *Achromobacter xylosoxidans* strain GH9OP genomic DNA as template, was conducted with GeneAmp PCR system 400 thermal cycler (PerkinElmer, Norwalk, Connecticut, USA). F-oph and R-oph primers were designed to contain, respectively, a *Bam* HI restriction site at *oph* start codon and *Hind* III restriction site at the stop codon (underlined bases) to facilitate cloning. 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^{o}C for three minutes as the initial denaturation step, followed by thirty-five cycles of denaturation at 94^{o}C for thirty seconds, annealing at 55^{o}C for one minute and extension at 72°C for one minute, and a final extension step at 72^{o}C for ten minutes. The anticipated PCR product of 950 bp was isolated after agarose gel electrophoresis using a gel extraction kit (Qiagen, Germany). The amplified fragment was cloned into the pTZ57R/T cloning vector (Thermo Scientific, Germany), and was transformed into *E. coli* XL1-Blue. Standard procedures for restriction endonuclease digestions, agarose gel electrophoresis, DNA ligation, and other cloning- related techniques were used as described by Sambrook and Russell (2001). Plasmids from transformant colonies were purified using Mini Plasmid Kit (Qiagen, Germany). The purified recombinant vector was sent to Clinilab, in Egypt for sequencing using vector primers. The obtained sequence was compared to other known sequences found in the Genbank by using the Blast program (http://www.ncbi.nlm.nih.gov/BLAST/)

2.6. Ethylmethane Sulfonate (EMS) Mutation Induction of the Achromobacter xylosoxidans strain GH9OP and Mutant Selection

The first-step EMS mutation induction was done as follows: one ml of an eighteen-hour old culture was centrifuged at 12,000 rpm for five minutes. The pellet was washed with and dissolved in 1mL of a 100 mM sodium phosphate buffer pH 7. 100 µL of the sample content was withdrawn to determine the initial population (CFU/mL). 20 µL/mL of the EMS stock solution 1gm/ml (Merck) was added to the samples in the falcon tubes for different times twenty, forty and sixty minutes, and was then incubated at 30°C. The reaction was stopped by the addition of 4 mL of sodium thiosulfate (5 %). The reaction was centrifuged; the pellets were washed and resuspended in a sodium phosphate buffer. Portions of 0.1 mL of suitable dilutions were spread on LB agar plates and incubated at 30°C for forty-eight hours. The bacterial colonies developed after incubation were counted and the survival percentages were estimated for each treatment (Verma *et al.*, 2016). Second-step EMS mutation induction was employed for the best mutant which resulted from first-step EMS mutation induction in the same manner illustrated earlier. In this study, the results of improved mutants are directed primarily based on their potential in the chlorpyrifos biodegradation compared to their wild strain.

3. Results

3.1. Evaluation of Chlorpyrifos Biodegradation by the Achromobacter xylosoxidans Strain GH9OP

Achromobacter xylosoxidans strain GH9OP showed effective activity in chlorpyrifos degradation. Figure 1 shows chlorpyrifos biodegradation by the *Achromobacter xylosoxidans* strain GH9OP after five, ten, and fifteen days; this bacterial strain was able to degrade 34.72 % after five days and 79.43 % after fifteen days of chlorpyrifos compared 1.03 % after five days and 3.05 % after fifteen days in the control media.

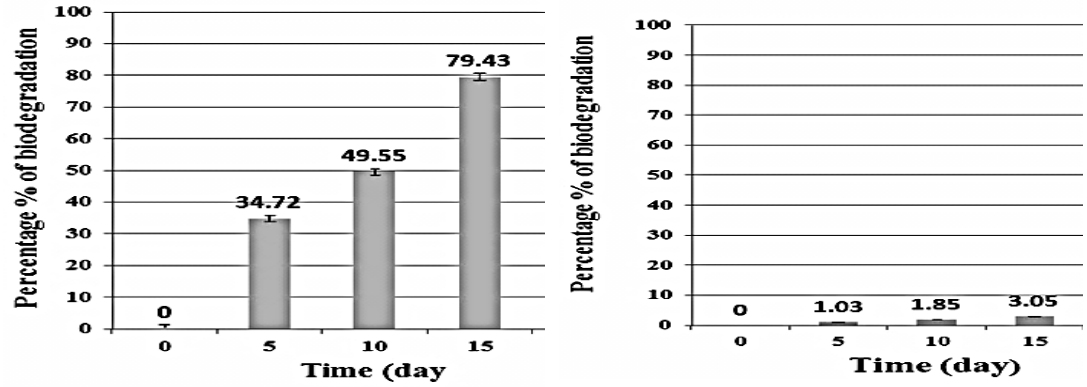

Figure 1. Biodegradation percentage of chlorpyrifos (480 mg/l) by *Achromobacter xylosoxidans* strain GH9OP (A). Degradation percentage of chlorpyrifos (480 mg/L) in control media (B).

3.2. Cloning and Complete Sequencing of the oph Gene in the Achromobacter xylosoxidans strain GH9Op

Primers, F-oph and R-oph containing *Bam* HI and *Hind* III restriction sites were used to amplify the complete coding sequence of the *oph* gene with a size of ~ 950 bp (Figure 2).

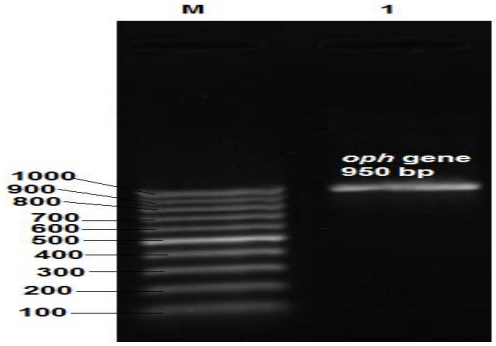

Figure 2. Agarose gel electrophoresis of PCR amplification for the oph gene in the Achromobacter xylosoxidans strain GH9OP (1). M: 100 bpDNA ladder (Jenabio).

The amplified fragment was cloned into pTZ57R/T cloning vector, and transformed into *E. coli* XL1-Blue. The new construct of pTZ57R/T vector carrying *oph* was termed as pTZ57R-oph.

Screening of transformant colonies with the recombinant vector and those containing non-recombinant vector was estimated by rapid screening of Plasmids using plasmid mini prep (Qiagen, Germany) (Figure 3), colony pcr using F-oph and R-oph (Figure 4) and Double digestion of pTZ57R-oph using *Bam* HI and *Hind* III endonuclease (Figure 5).

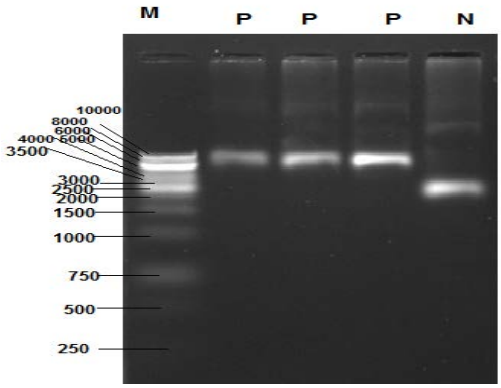

Figure 3. Agarose gel electrophoresis of isolated plasmid from transformant E. coli XL1-Blue. P: transformant colonies containing pTZ57R-oph vector. N: transformant colonies containing pTZ57R vector only.

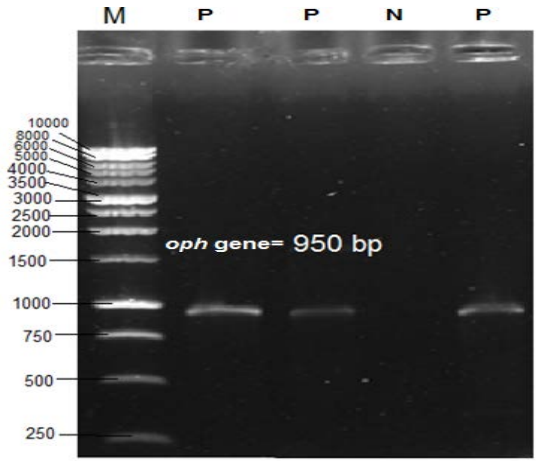

Figure 4. Agarose gel electrophoresis for colony PCR products of three colonies of *E. coli* XL1-Blue transformed with the recombinant plasmid pTZ57R-oph containing *oph* gene from *Achromobacter xylosoxidans* strain GH9OP by using *oph* gene primers. (M): 1 Kb DNA ladder (Jenabio). P: Positive colony in pcr amplification of *oph* gene. N: Negative colony in PCR amplification of *oph* gene.

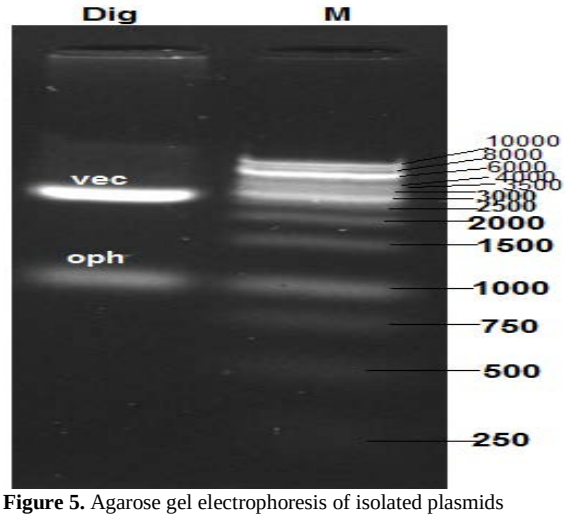

Figure 5. Agarose gel electrophoresis of isolated plasmids digested by *Bam* HI and *Hind* III restriction enzymes (Dig). (M): I Kb DNA ladder (Jenabio).

The DNA sequence of the *oph* gene was subjected to a search on (https://www.ncbi.nlm.nih.gov/ Structure/ cdd/ wrpsb.cgi), and the result was that a conserved domain in the isolated *oph* gene is a member of the MBL-fold metallo hydrolase superfamily and has a beta lactamase fold. It has a 99 % similarity with that of *Achromobacter xylosoxidans* NH44784-1996 Accession no. HE798385.1. Deduced protein sequences of both genes were aligned using CLUSTAL multiple sequence alignment, MUSCLE 3.8 (Figure 6). The *oph* gene in *Achromobacter xylosoxidans* GH9OP was firstly isolated by the authors of this study from Egyptian soils, and the DNA sequence of the *oph* gene was deposited in Genbank database under the accession number MH018244.

```
MH018244.1                MPSATQTLPFSSLSDPCVRPPHDLVPSRYALRVGEIDALVISDGVLPLPT
HE798385.1_1841224-1842159 MPSATQTLPFSSLSDPGVRPPHDLVPSRYALRVGEIDALVISDGVLPLPT
                          *************** ************************************

MH018244.1                ATMATNADPADLARWLQYMFMPPDAFDWPLNVMVARSGDQTILIDAGLGG
HE798385.1_1841224-1842159 ATMATNADPADLARWLQYMFMPPDAFDWPLNVMVARSGDQTILIDAGLGG
                          ************************************************

MH018244.1                QFPGFPRAGQLPQRLEDAGIALESVTDVIITHMHMDHVGGLLVDGVKERL
HE798385.1_1841224-1842159 QFPGFPRAGQLPQRLEDAGIALESVTDVIITHMHMDHVGGLLVDGVKERL
                          ************************************************

MH018244.1                RPDVRIHVSATEVAFWTSPDFSHTVMPKPVPAVLRSTAASFYNEYRDRLR
HE798385.1_1841224-1842159 RPDVRIHVSATEVAFWTSPDFSHTVMPKPVPAVLRSTATSFYNEYRDRLR
                          ************************************:***********

MH018244.1                IFQDRHEVAPGVVVRITGGHTPGHSVVDLIGGDERLTFAGDAIFPVGFDH
HE798385.1_1841224-1842159 IFQDRHEVAPGVVVRITGGHTPGHSVVDLIGGDERLTFAGDAIFPVGFDH
                          ************************************************

MH018244.1                PDWHNGFEHDPEESARVRLRLFQELAQNRGLLVAAHLPFPSVGRVAIDGD
HE798385.1_1841224-1842159 PDWHNGFEHDPEESARVRLRLFQELAQNRGLLVAAHLPFPSVGRVAIDGE
                          ************************************************:

MH018244.1                AFRWVPVIWDY
HE798385.1_1841224-1842159 AFRWVPVIWDY
                          ***********
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Figure 6. Pairwise alignment of deduced amino acid sequences of the *oph* gene from the *Achromobacter xylosoxidans* strain GH9OP accession no. MH018244 and the organophosphorus hydrolase enzyme (*oph)* gene from *Achromobacter xylosoxidans* NH44784-1996 Accession no. HE798385.1:1841224-1842159.

3.3. EMS Mutation Induction in Achromobacter xylosoxidans GH9Op

3.3.1. First-Step EMS Mutation Induction in Achromobacter xylosoxidans GH9Op

The Mutagenesis approach was widely used to improve the production of various microbial enzymes (Hussein *et al.*, 2012). It is the first time to improve the organophosphorus hydrolase enzyme responsible for chlorpyrifos biodegradation by random mutation using ethyl methanesulfonate (EMS) as a chemical mutagen. After the induction of EMS mutation with the *Achromobacter xylosoxidans* strain GH9OP for different treatment periods, viable colonies were counted from appropriate dilution. The survival percentage was calculated and the results are presented in Figure 7. It was noticed that the survival percentage decreased as the treatment time increased due to the lethal effect of EMS. The least survival percentage was recorded at an exposure period of forty minutes. The exposure period of sixty minutes was lethal for the wild strain.

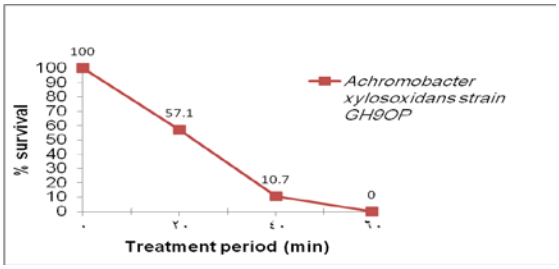

Figure 7. Survival percentage of *Achromobacter xylosoxidans* strain GH9OP after EMS (20 µL/mL) mutation induction.

3.3.1.1. Effect of EMS Mutation Induction on the Biodegradation of Chlorpyrifos by Achromobacter xylosoxidans GH9OP

In spite of the fact that genetic engineering has made a significant contribution to the improvement of bacterial strains, random mutagenesis is still a cost-effective procedure for reliable short-term strain development and is frequently preferred as the method of choice (Lipika, 2014). EMS is a well-known chemical mutagenic agent whose mode of action is attributed to the alkylation of nitrogen position 7 of guanosine of the DNA molecule causing G/C to A/T transition mutation (Freese, 1961).

After a twenty-minute treatment by EMS, twenty-nine mutants were selected, twenty-one mutants exhibited approximately equal activity to the wild type, and eight mutants exhibited higher activity compared to the wild type. The highest biodegradation was recorded by AchM12 and AchM15 which were able to degrade 63 % of chlorpyrifos compared to 34 % in the wild type. After forty minutes of the EMS treatment, sixteen mutants were selected; seven mutants exhibited less activity than the wild type, three mutants exhibited approximately equal activity to the wild type, and only four mutants exhibited a higher activity than the wild type especially AchM44 which degraded 49 % chlorpyrifos. All biodegradation activity was recorded after five days (Table 1) .

Table 1. Chlorpyrifos biodegradation by EMS-induced mutants from the *Achromobacter xylosoxidans* strain GH9OP after five days

Treatment exposure time (min)	Bacterial isolates	*dry weight g/100 mL	*% chlorpyrifos biodegradation	Bacterial isolates	*dry weight g/100 ml	*% chlorpyrifos biodegradation
20	wild type	0.281	34.23	AchM15	0.289	63.41
	AchM1	0.290	33.90	AchM16	0.269	34.08
	AchM2	0.271	33.90	AchM17	0.290	34.01
	AchM3	0.273	34.50	AchM18	0.289	34.90
	AchM4	0.254	36.89	AchM19	0.278	41.95
	AchM5	0.270	33.99	AchM20	0.301	33.99
	AchM6	0.291	36.07	AchM21	0.258	33.98
	AchM7	0.287	37.13	AchM22	0.294	37.91
	AchM8	0.279	33.99	AchM23	0.290	36.93
	AchM9	0.269	33.98	AchM24	0.286	33.00
	AchM10	0.269	34.09	AchM25	0.293	34.08
	AchM11	0.291	34.08	AchM26	0.310	37.30
	AchM12	0.276	63.20	AchM27	0.297	34.70
	AchM13	0.293	33.89	AchM28	0.311	34.70
	AchM14	0.287	33.44	AchM29	0.264	34.49
40	AchM30	0.201	33.97	AchM38	0.08	1.13
	AchM31	0.253	36.90	AchM39	0.188	19.80
	AchM32	0.290	34.09	AchM40	0.299	42.00
	AchM33	0.281	34.10	AchM41	0.190	14.97
	AchM34	0.063	0.93	AchM42	0.289	42.98
	AchM35	0.191	15.09	AchM43	0.179	22.13
	AchM36	0.189	21.50	AchM44	0.301	49.00
	AchM37	0.193	20.69	AchM45	0.180	18.16

*Mean of three replicates

3.3.2. Second-Step EMS Mutation Induction

The highest mutant, AchM15, in the chlorpyrifos biodegradation, resulted from the first-step EMS mutation induction for the *Achromobacter xylosoxidans* strain GH9OP for twenty minutes which was then mutated by EMS under the same conditions previously employed for the wild bacterial isolates. The highest mutant (AchMS1) was obtained after the second-step EMS treatment of AchM15 for forty minutes; it recorded a higher biodegradation potential than AchM15. It degraded 82.03 % of chlorpyrifos compared with 65 % by AchM15 after five days.

This study achieved improved OPH (organophosphorus hydrolase) variants with increased activity towards poorly-hydrolysed substrates such as chlorpyrifos by random mutation using the EMS-treatment approach which leads to an enzymatic solution. Figure 8 shows the growth of the wild type, *Achromobacter xylosoxidans* strain GH9OP and its mutants, AchM15 and AchMs1. All were grown in minimal media containing chlorpyrifos with the aforementioned concentration.

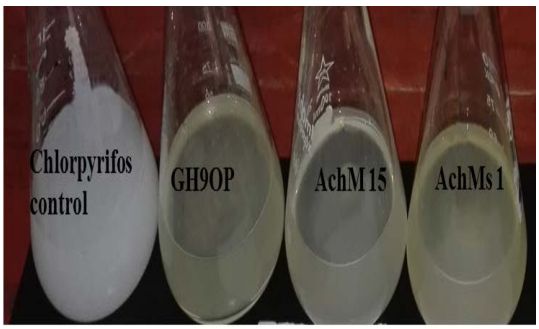

Figure 8. Bacterial growth of the *Achromobacter xylosoxidans* strain GH9OP and its mutants on liquid MSM containing Chlorpyrifos (480 mg/l) after 5 days. GH9OP: *Achromobacter xylosoxidans* wild strain. AchM15: mutants from first-step EMS mutation induction. AchMs1: mutant from second-step EMS mutation induction. Chlorpyrifos Control: liquid MSM containing chlorpyrifos without bacterial inoculum.

3.3.3. Evaluation of the Behavior of Achromobacter xylosoxidans GH9OP and Their Higher Mutants in OPs Degradation through Time Shift

The biodegradation of chlorpyrifos by *Achromobacter xylosoxidans* GH9OP as a wild strain and its selected higher mutants, AchM15 and AchMS1 were identified after five, ten, and fifteen days. Figure 9 shows that the periods of five and ten days were the perfect periods at which the highest biodegradation occurred in both wild bacterial strain and its mutants. These periods were the transformation points at which difference between wild strains and their mutants was emphasized. The *Achromobacter xylosoxidans* strain GH9OP degraded 54.22 % of chlorpyrifos and its mutants. AchM15 and AchMs1 degraded 75.08 % and 92.03 %, respectively compared with 7.14 % in case of control after ten days. It can be included that the biodegradation achieved by the wild strain over fifteen days could be achieved in five days by their higher mutants. All treatments were carried in triplicate.

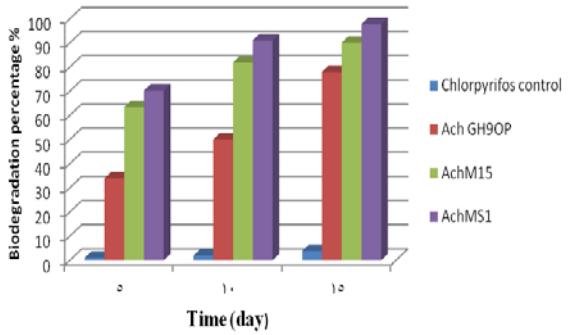

Figure 9. Chlorpyrifos biodegradation by *Achromobacter xylosoxidans* and its mutants**.**

3.4. Identification of the Degradation Products of Chlorpyrifos in Achms1 by GC/MS

In this study, it is the first time to manipulate the bacterial mutant, AchMS1, in pesticides' biodegradation so that it can be used as a model strain to detect chlorpyrifos metabolites. The metabolic products of chlorpyrifos degradation were confirmed by GC-MS. The results 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 a retention times (RT) of 30.03 minutes. This peak disappeared concomitantly with the formation of other new peaks with a retention time of around 24.1 minutes (Figure 10). Each peak was identified on the basis of its mass spectra and the NIST library identification program. The degradation pathway for chlorpyrifos by AchMS1 was proposed in Figure 11. That is to say, the parent chlorpyrifos (m/z = 351) was first metabolized by hydrolysis to produce TCP (m/z = 197) and diethyl thiophosphoric acid (DETP) (m/z = 172). Subsequently, the hydrolysis product, TCP was further transformed by a ring breakage, resulting in its detoxification. These results indicated that chlorpyrifos (480 mg/L) was degraded by AchMs1 without any accumulative products after ten days of incubation.

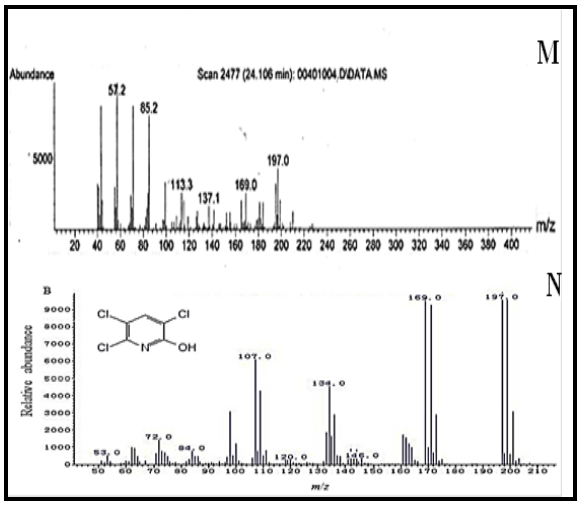

Figure 10. Mass spectra of 3, 5, 6-trichloro-2-pyridinol (TCP) produced from chlorpyrifos degradation by AchMS1. M: sample; N: authentic standard TCP from the National Institute of Standards and Technology (NIST, USA) library database.

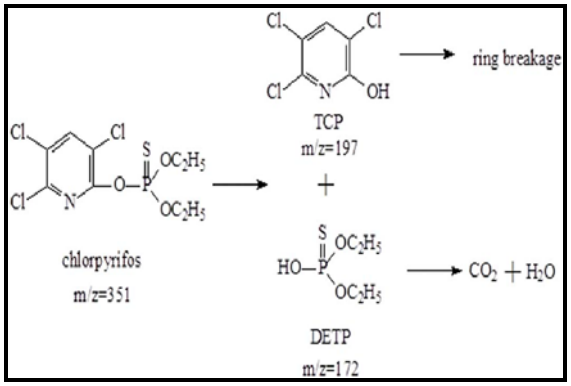

Figure 11. The proposed pathway for the chlorpyrifos biodegradation by AchMS1.

4. Discussion

The *Achromobacter xylosoxidans* strain, GH9OP, showed a high potential in chlorpyrifos biodegradation, This result is inconsistent with the previous reports that employed the *Achromobacter xylosoxidans* (JCp4) and *Ochrobacterum sp.*(FCp1) in chlorpyrifos biodegradation. It was found that these strains were able to degrade chlorpyrifos in non-sterilized and sterilized soils and exhibited the ability to degrade 93 % to 100 % of the input concentration 200 mg/l within forty-two days (Akbar and Sultan, 2016). In this study, the *Achromobacter xylosoxidans* strain GH9OP is a promising candidate for raising the productivity of crops in pesticide- contaminated soils. Many bacterial strains are involved in organophosphorus pesticides' degradation. Among these bacteria are: *Enterobacter* sp. (Singh *et al.*, 2004), *pleismonas* sp. (Zheng *et al.*, 2013), *Agrobacterium radiobacter* (Horne *et al.*, 2002), and *Streptomyces* sp. (Nelson, 1982). Different bacterial genes are responsible for the organophosphates degradation, and these genes exhibit different DNA sequences in different bacterial strains. For example, Serdar *et al.* (1982) isolated the first described organophosphorus degrading the *opd* gene from *P. diminuta,* and Mulbry *et al.* (1987) isolated the *opd* gene from the *Flavobacterium* sp. strain, ATCC 27551.

Significant homology between *opd* genes from the two bacteria was demonstrated by Southern hybridization experiments (Mulbry *et al.*, 1987). *opa*A gene is another organophosphorus-degrading gene which has received considerable attention. It was isolated and cloned from *Alteromonas* sp. JD6.5 by Cheng *et al.* (1997). Despite, functional similarity with the *opd* gene, no sequence homology was found between them. Zhongli *et al.* (2001) isolated a methyl parathion degrading the *mpd* gene from the *Plesiomonas* sp. strain M6. The sequencing and cloning of this gene revealed that no region of extensive DNA homology was observed between the *mpd* gene and those in the Genbank database. A 31 % similarity between protein sequence and beta-lactamase was found, suggesting the significant novelty of the gene-enzyme system.

According to the analysis of DNA sequence of the *oph* gene in the *Achromobacter xylosoxidans* GH9OP by using conserved domains on NCBI, (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), this gene is a member of the MBL-fold metallo hydrolase superfamily, and has a beta lactamase fold which is mainly hydrolytic enzymes involved in the organophosphates hydrolysis (Bigley and Raushel, 2013). Salman *et al.* (2010) reported that a total of sixty different enzymes involved in organophosphate degradation are divided into eight subgroups based on the type of organism. 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. Consequently, they give rise to a different active site structure.

Site-directed mutagenesis as a tool to manipulate Catalytic mechanism and enzyme properties was successfully used to enhance the activity of OPH against racemic mixtures of organophosphorus enantiomers. Wu *et al.* (2001) and Raushel, (2002) used site-directed mutagenesis for remoulding the size and shape of the substrate binding subsites through rational restructuring. However, due to unexpected influences exerted by substituted amino acids, rational designs can fail sometimes. It is the first time in this study to use EMS-mutation induction as an effective and cost-effective technique to improve the bacterial organophosphorus hydrolase enzyme activity towards poorly degradable substrates such as chlorpyrifos. The obtained higher mutants such as AchM15 and AchM12 exhibited a cell mass that was slightly equal to that of the wild type; this means that the increase in the biodegradation activity is related to the increase in the gene-enzyme expression. In the highest mutants, the increase in the chlorpyrifos biodegradation may be attributed to several reasons including the overexpression of the gene encoding the enzyme (organophosphorus hydrolase enzyme) responsible for the OPs biodegradation, which increases the efficiency of the secretion system. Changes in one or more amino acids of the enzyme result in more binding efficiency between the produced enzymes and their substrates leading to more biodegradation activity. EMS is able to induce the overproduction of other enzymes including α-amylase, xylanase, cellulase and laccase indifferent bacterial strains. When Haq *et al.* (2009) treated *Bacillus licheniformis* with EMS to improve the α-amylase production, the best mutant increased was by about 39 % compared to the wild type Strain. Hanim *et al.* (2013) improved xylanase production from the xylanolytic bacteria using EMS, and the specific activity was increased by 61.61 % from 9.317 U/g to 15.057 U/g. Narasimhan *et al.* (2013) used EMS to improve the cellulase activity of *Bacillus subtilis*, and mutant M57 showed 4.71 fold increasing in cellulase activities. Verma *et al.* (2016) employed EMS to improve the the production of laccase from *Pseudomonas putida*, LUA15.1, isolated from rice rhizospheric soil samples of paddy fields, in Himachal Pradesh (India). They obtained mutant E4 (34.12 U/L) resulting in a 26.37 % increase in the laccase activity compared to the wild strain (25.12 U/L). However, mutants, AchM34 and AchM38 showed no biodegradation activity, and this may be attributed to the damage of the structure gene where mutation changes in the structure genes most probably result in the loss of function (Ho and Chor, 2015). Their weak growth in the minimal media containing chlorpyrifos as a carbon source was due to their inability to produce the organophosphorus hydrolase enzyme responsible for the chlorpyrifos biodegradation and unused produced metabolites as a carbon source.

Vijayabaskar *et al.* (2014) used the best UV mutant of the *Bacillus pumilus* strain to further improve the Carboxy methyl cellulase by EMS. This treatment improved the activity by 96.24 %. Taking the same path, this study obtained AchMS1 from the EMS mutation second-step for AchM15. The increase in the biodegradation potential in AchMS1 may be attributed to stabilizing the structure of the enzyme and promoting the interactions between the enzyme and its substrate. Moreover, when a microbial culture is exposed simultaneously to mutagenic agents, the frequency of mutation increases. The duplication of the gene that occurred enables it to increase the inefficient enzyme production. As a result, mutations may occur in the enzymes' structural gene, enabling the former and inefficient enzymes to metabolize their substrates more efficiently and effectively (Monroe, 2005). It can be concluded that chemical mutagenic agents that possess the ability to induce mutation have been a major driving force in the field of genetic studies for the past seventy-five years (Muller, 1930).

This study generated potent OPs-degrading bacterial mutants contributing to increasing in OPS detoxification. AchMS1 exhibited the ability to degrade chlorpyrifos into non-polar compounds. This feature is rarely reported in other chlorpyrifos-degrading microorganisms. In most cases reported to date, the individual isolate is able to transform chlorpyrifos by hydrolysis of ester linkage to give TCP, which in turn accumulates in the batch cultures or soils. Moreover, enhanced degradation could not occur due to its antimicrobial properties (Anwar *et al.*, 2009; Li *et al.*, 2010). TCP has an estrogenic activity and has recently been listed as a potential endocrine disrupting chemical by the Environmental Protection Agency (EPA, 2009) of the USA. Compared with the importance of TCP degradation issue, studies concerning its degradation and fate in the environment are very limited. Chen *et al.* (2012) isolated a new fungal strain Hu-01 identified as *Cladosporium cladosporioides* with a high chlorpyrifos-degradation activity and utilized 50 mg/l of chlorpyrifos as the sole carbon source at acidic pH 6.5. This study is in contrast with his previous findings, because chlorpyrifos biodegradation by the bacterial mutant in this study was

perfect in a basic pH that is already present in most Egyptian agriculture soils. Another important feature worth mentioning is that the bacterial mutant under study engaged in efficient degradation of chlorpyrifos at a high concentration in contrast to other reports on the toxic effects of OPs in diverse microorganisms (Racke *et al.*, 1990; Mallick *et al.*, 1999). The high chlorpyrifos tolerance and degradation capability of the bacterial mutant make this mutant suitable for decontamination and bioremediation of the Ops- contaminated sites.

5. Conclusion

The present study investigated the ability of the *Achromobacter xylosoxidans* strain GH9OP in chlorpyrifos biodegradation. This strain exhibited a high chlorpyrifos biodegradation activity. The *oph* gene encoding the organophosphorus hydrolase enzyme responsible for chlorpyrifos biodegradation was cloned, and the ORF of this gene was sequenced for the first time. EMS mutation induction is an effective approach in the enhancement of the chlorpyrifos biodegradation activity. This study confirms the safe application of the AchMS1 bacterial mutant in chlorpyrifos bioremediation issues due to the production of metabolites which are less toxic than the parent substrate.

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