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ISOLATION AND MOLECULAR IDENTIFICATION OF INDIGENOUS BACTERIAL ISOLATES ABLE TO DEGRADE ORGANOPHOSPHATES

[184]

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ABSTRACT

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 degradation of pesticides in contaminated soils has been considered advantageous to decontaminate areas that have been polluted by pesticides. Chlorpyrifos and diazinon were the most persistent residues in Egyptian soils. Four bacterial isolates were isolated from organophosphorus insecticides contaminated soils and genetically identified based on DNA sequence of 16s rDNA gene, *Cronobacter muytjensii* GH10, *Achromobacter xylosoxidans* GH9OP, *Pseudomonas aeruginosa* GH2NO8 and *Pseudomonas putida* GH4SNO/P were able to degrade 92.59%, 97.75%, 91.82%, and 90.78% of diazinon (600mg/l) as compared with 16.99% in control and 93.43%, 78.51%, 93.18% and 95.36% of chlorpyrifos (480mg/l) as compared with 4.28%, in control, respectively after 20 days of incubation.

Keywords: Organophosphates, biodegradation, 16sr DNA gene, Gas chromatography analysis.

INTRODUCTION

The pesticides extensive use through accidental spills, handling, crop spraying, rinsing of containers, etc. has potential to contaminate soil, air and water (**Akbar and Sultan 2016).** 38 percent 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 **(Dhanya 2014)**

Although (Ops) play important roles in protecting agriculture crop from weeds , insect pests and in disease-transmitting vectors controlling, they irreversibly in activate acetylcholine esterase (AChE) which is essential to nerve function in insects, human and many other animals. Inhibition of acetylcholine esterase results in neurotransmitter acetylcholine accumulation and acetyl choline receptor continued stimulation such; they could cause acute or chronic poising in human being (**Zajic and Supplisson 1972)**.

Chemical treatments, incineration and landfills are the current methods in OPs detoxification **(Salman et al 2010)**. Incineration although the most reliable method for OPs destructions, it has involved a serious public opposition due to the potentially toxic emission **(Richins et al 1997)**. Chemical methods are problematic due to production of large volumes of acids and alkali which 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 **(Ang et al 2005)**. 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 **(Xie et al 2013)**. For example the hydrolysis of parathion resulted in 100-fold reduction in toxicity **(Lan et al 2006).**

Various organophosphorus pesticides degrading bacteria able to use OPs as carbon, nitrogen as an energy source were isolated from pesticides contaminated environments **(Hsu et al 2008)**. Pesticide degrading bacteria are including *Flavobacterium sp.* **(Mulbry and Karns 1989)**, *Pseudomonas diminuta* MG **(Gilani et al 2016)**, *Penicillium lilacinum* BP **(Liu et al 2004)**, *Arthobacter sp*. **(Ohshiro et al 1999)**. These bacterial strains have the ability to degrade OPs by different types of enzymes. The most characterized enzymes are phosphotriesterase (PTEs). 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 **(Hill 2003)**

The goals of this study were to isolate and identify new bacterial strains from OPS contaminated Egyptian agriculture soils able to degrade and use 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.

MATERIALS AND METHODS

Reagents and Chemicals

Analytical grade chlorpyrifos (48%) and diazinon (60%) emulsifiable concentrate were purchased from (Sinochem Agro.Co.Ltd, China) .All reagents and solvents used in the present study were of analytical grade. Diazinon and chlorpyrifos were used as organophosphorus insecticides models due to their high residues in Egyptian soils **(Metwally 2014).**

Detection the most persistent organophosphorus pesticides in the Egyptian soils

Soil samples from agriculture fields in different governorates, Sharqia, Minufiyah, Qalyubiyah and Giza, Egypt were screened for the purpose of detection of the most persistent pesticides residues by Gas Chromatography analysis in Central Agriculture Pesticides Lab. (CAPL), Agriculture Research Center. Giza, Egypt.

Enrichment and selection of pesticides degrading bacterial isolates

Soil samples were transferred to the laboratory and first enriched with access of chlorpyrifos and diazinon which were detected in Egyptian soils in high residues for 10 days after thorough mixing to isolate potent bacterial isolates in organophosphorus pesticides biodegradation. Further enrichments were then carried out in which one gram from previously enriched soils was used to inoculate 50 ml autoclaved minimal salt media (MSM) containing 600 mg/L diazinon and 480 mg/L chlorpyrifos as a sole carbon source **(Singh 2009)**. Cultures were then incubated on an orbital shaker at 30^{o}C for 5 days at 150 rpm.

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

Degradation of chlorpyrifos and diazinon in minimal salt liquid media

The bacterial isolates were transferred to MSM media containing diazinon (600 mg/l) and chlorpyrifos (480 mg/l) as a sole carbon source. Each sample was prepared in triplicate. All cultures were incubated at 30^{o}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 after 0, 5, 10, 15 and 20 days of incubation, the residual diazinon and chlorpyrifos were determined in the culture extract of the bacterial isolates and measured using gas chromatography (GC) analysis **(Dhanya 2014)**.

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 18h. The cultures were centrifuged at 13,000 rpm for 5min at 4^{o}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 gene.

Molecular identification of bacterial isolates by PCR amplification and sequencing of 16S rDNA gene

Molecular tools such as 16S rDNA pcr amplification were used to identify the bacterial isolates **(Hoffman et al 2010)**. Bacterial 16S rDNA was amplified by PCR using the universal primers; forward primer sequence (5´AGAGTTTGATCCT GGCTCAG3´) and reverse primer sequence (5´CTACGGCTACCTTGTTACGA3´) thereby producing an amplicon of ~1500 bp. Amplification was carried out in 50µl 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^{o}C for 3 min as initial denaturation step followed by 35 cycles of denaturation at 94^{o}C for 30 sec, annealing at 55^{o}C for 1 min, extension at 72^{o}C for 2 min and final extension at 72^{o}C for 10 min. PCR product was purified by QIAquick Gel Extraction Kit (Qiagen, Germany) following the protocol provided by the supplier and then resolved by electrophoresis on 1.5 % agarose gel. 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.

RESULTS AND DISCUSSION

Detection the most persistent pesticides residues in the Egyptian soil samples

In soil samples under study, Diazinon and chlorpyrifos as organophosphorus pesticides showed higher concentrations than that of other detected pesticides such as Oxamyl and pirimiphos methyl. Chlorpyrifos recorded 0.07 mg/kg soil in Beheira and Minufiyah but it recorded 0.09 mg/kg soil in Qalyubiyah. Diazinon concentration ranged between 0.008mg/kg soil in Kaliopia to 0.08 mg/kg soil in Beheira **Table (1)**. These results were agreed with **Metwall (2014)**, he discovered that organophosphorus pesticides, diazinon and chlorpyrifos were the most persistent residues in different Egyptian soils.

Table 1. The concentrations of pesticides residues in soil samples

Soil sample locations	Detected pesticides residues	Concentration/kg soil
Giza	Chlorpyrifos	0.01
	Oxamyl	0.002
	Adicarb	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

Isolation of potent organophosphorus degrading bacterial isolates

Few bacterial isolates capable of degrading organophosphorus pesticides have been reported till date. Four different aerobic bacterial isolates capable of degrading chlorpyrifos of initial concentration (480 mg/l) and diazinon of initial concentration (600 mg/l) by the enrichment technique were developed from five soil samples collected from various agricultural fields. These four bacterial isolates labeled as, GH10, GH2NO8, GH9OP and GH4SNO/P have showed good growth on minimal salt media containing diazinon and chlorpyrifos with concentrations mentioned previously after 5 days at pH 8 **Fig. (1, 2)**.

Fig. 1. Growth of bacterial isolates on liquid minimal salt media containing chlorpyrifos (480 mg/l) after 5 days incubation. GH9OP, GH2NO8, GH10 and GH4SNO/P were the bacterial isolates; Control: liquid MSM with chlorpyrifos (480 mg/l) without bacterial inoculums

Fig. 2. Growth of bacterial isolates on liquid minimal salt media containing diazinon (600 mg/l) after 5 days incubation. GH9OP, GH2NO8, GH10 and GH4SNO/P are the bacterial isolates; Control: liquid MSM with diazinon (600 mg/l) but no bacterial inoculums.

Molecular identification of OPs degrading bacterial isolates by PCR amplification of the 16S rDNA

16s rDNA gene universal primers amplify ~1550 bp for all bacterial isolates (Fig 3). Partial sequences were analyzed, bacterial isolate GH10 16s rDNA gene partial sequence has 99% similarity with that of *Cronobacter muytjensii* strain ATCC 51329 accession no. CP012268. Bacterial isolate GH10 was named as *Cronobacter muytjensii* strain GH10. 16s rDNA gene partial sequence of GH2NO8 bacterial isolate has 100% similarity with that of *Pseudomonas aeruginosa* strain GHJ12 accession no. MG396955. GH2NO8 bacterial isolate was identified as *Pseudomonas aeruginosa* strain GH2NO8. 16s rDNA gene partial sequence of bacterial isolate GH9OP has 100% similarity with that of *Achromobacter xylosoxidans* strain NCCP-44 accession no. AB547225 Thus it was identified as *Achromobacter xylosoxidans* strain GH9OP. With respect to the GH4SNO/P bacterial isolate, its 16s rDNA gene partial sequence has 99% similarity with that of *Pseudomonas putida* strain JR4 accession no. KY982927. This strain was named as *Pseudomonas putida* strain GH4SNO/P. 16s rDNA genes partial sequences of bacterial isolates were deposited in the GenBank database with accession numbers shown in **Table (2).**

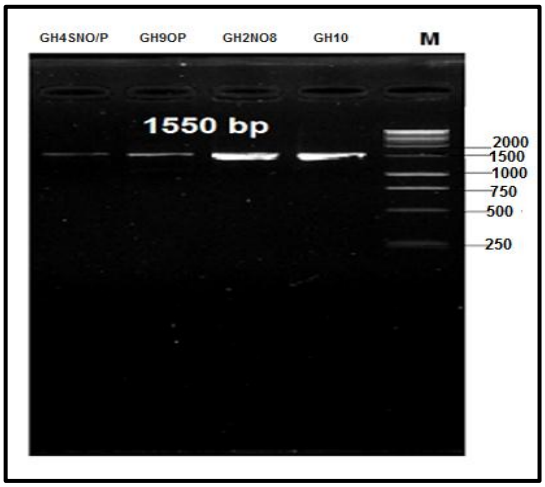

Fig. 3. Agarose gel electrophoresis for PCR product of 16s rDNA gene. M: 1Kb DNA ladder (Thermoscientific, Germany)

Table 2. Accession numbers of 16s rDNA gene of bacterial isolates

Bacterial isolates codes	Molecular identification	Accession number
GH10	*Cronobacter muytjensii* strain GH10	KY945346
GH2NO8	*Pseudomonas aeruginosa* strain GH2NO8	KY945349
GH9OP	*Achromobacter xylosoxidans* strain GH9OP	KY945347
GH4SNO/P	*Pseudomonas putida* strain GH4SNO/P	KY945348

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 after 20 days of incubation **Fig. (4)**. This search has been the first study that manipulates *Cronobacter muytjensii* in pesticides biodegradation where this bacterial isolate showed a remarkable biodegradation activity.

There was a considerable removal of diazinon and chlorpyrifos by *Pseudomonas aeruginosa* strain GH2NO8, *Achromobacter xylosoxidans* strain GH9OP and *Pseudomonas putida* strain GH4SNO/P. They were able to degrade 91.82%, 97.75% and 90.78% respectively of the former 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. (5, 6, 7)**. Diazinon and chlorpyrifos control biodegradation was shown in **Fig. (8)**. 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. These results are in consistent with the previous reports, where bacterial strain *Achromobacter xylosoxidans* (JCp4) and *Ochrobacterum sp*. (FCp1) were able to degrade chlorpyrifos in sterilized and non-sterilized soils , they exhibited the ability to degrade 93% to 100% of the input concentration 200 mg/l within 42 days **(Akbar and Sultan 2016)**. In this study, *Achromobacter xylosoxidans* strain GH9OP is a promising candidate for raising the productivity of crops in pesticides 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)**. These results are agreed with that obtained by **Khani and Kafilzadeh (2015)**, they isolated *Pseudomonas aeruginosa* and *Flavobacterium sp.* which were able to reduce the level of diazinon at level of $p \leq 0.05$. **Khalid et al (2016)** isolated bacterial strain *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 (2009)**. The metabolic specificity of OP degrading microorganisms, however, is determined by the chemical resemblance among the OP compounds. Both insecticides tested in this work have phosphotriester bond in their molecular structures, suggesting that hydrolysis at this bond takes place.

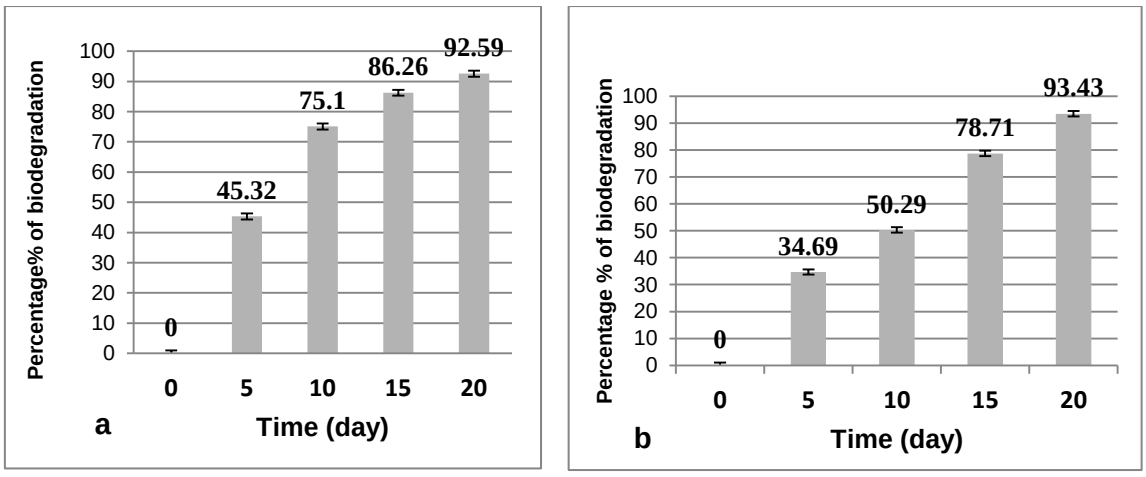

Fig. 4. (a): biodegradation Percentage of diazinon (600 mg/l) and (b): Biodegradation of chlorpyrifos (480 mg/l) by *Cronobacter muytjensii* strain GH10

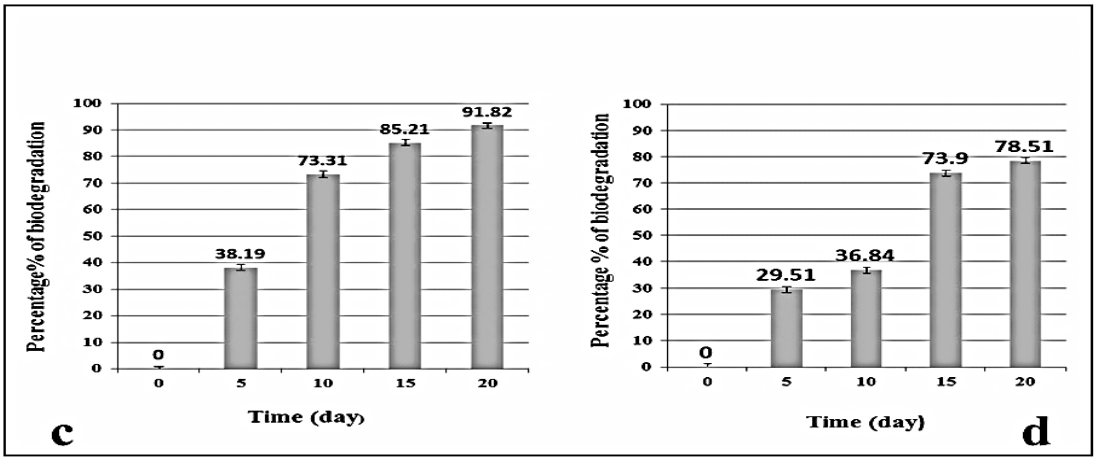

Fig. 5. (c): Percentage of biodegradation of diazinon (600 mg/l) and (d): Biodegradation of chlorpyrifos (480 mg/l) by *Pseudomonas aeruginosa* strain GH2NO8

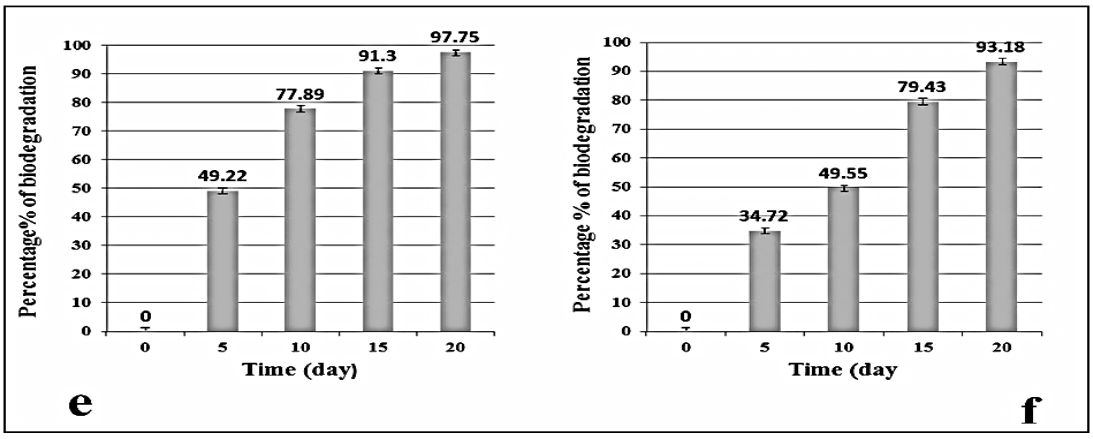

Fig. 6. (e): Percentage of biodegradation of diazinon (600 mg/l) and (f): Biodegradation of chlorpyrifos (480 mg/l) by *Achromobacter xylosoxidans* strain GH9OP

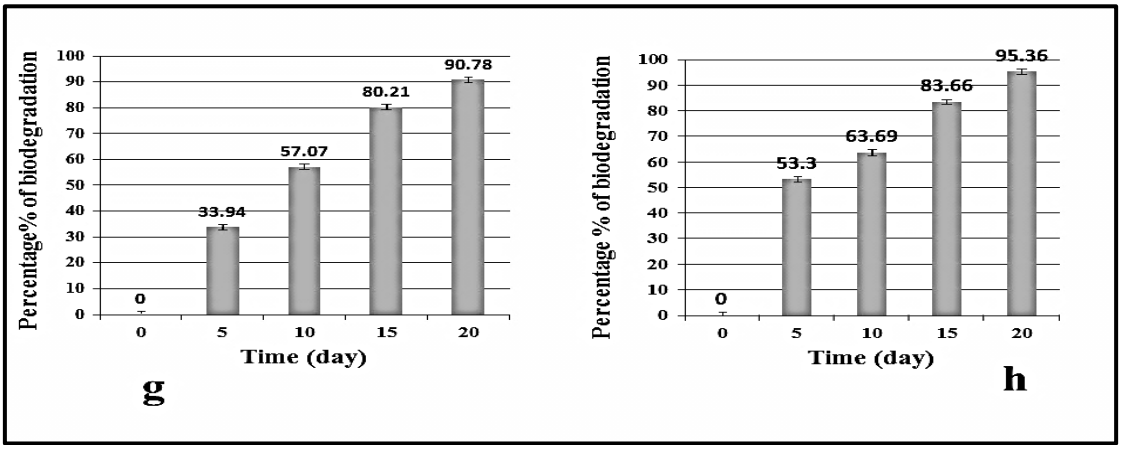

Fig. 7. (g): Percentage of biodegradation of diazinon (600 mg/l) and (h): Biodegradation of chlorpyrifos (480 mg/l) by *Pseudomonas putida* strain GH4SNO/P

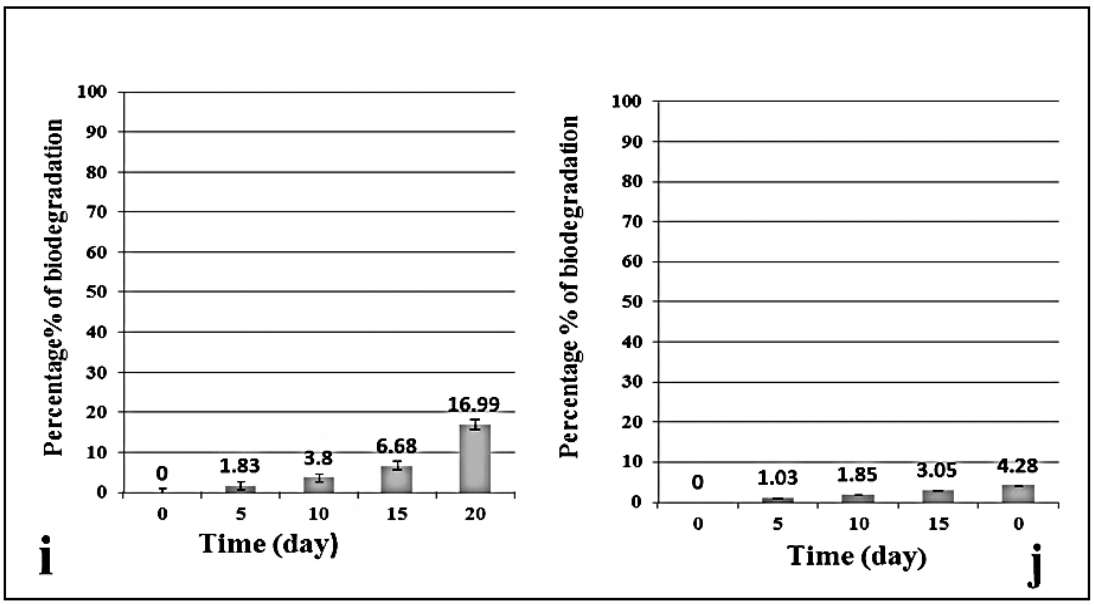

Fig. 8. (i): Percentage of biodegradation of diazinon (600mg/l) and (j): Biodegradation of chlorpyrifos (480 mg/l) in control media

CONCLUSION

Chlorpyrifos and diazinon represented the major organophosphorus pesticides residues in Egyptian soils. Four bacterial isolates, *Cronobacter muytjensii* strain GH10, *Pseudomonas aeruginosa* strain GH2NO8, *Achromobacter xylosoxidans* strain GH9OP and *Pseudomonas putida* strain GH4SNO/P improved high activity in organophosphates biodegradation and that makes them potent candidates in organophosphates detoxification in contaminated niches.

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عزل وتعريف جزيئي لعزلات بكتيرية محلية قادرة على التكسير الحيوى للمبيدات العضوية الفسفورية

[184]

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الموجـــــز

تم تحديد أكثر المبيدات تراكما فى أربعة أنواع مختلفة من التربة المصرية فى محافظات مختلفة (الشرقية – المنوفية – القليوبية– الجيزة) حيث وجد ان الديازينون والكلوربيريفوس كنموذجان للمبيدات العضوية الفسفورية من أكثر المبيدات تراكما فى التربة المصرية.

تم عزل أربعة عزلات بكتيرية لها قدرة هائلة فى تكسير تلك المبيدات. تم تعريف هذه العزلات عن طريق معرفة التتابع النيوكليوتيدى لجين 16sr DNA وبقارنة هذه التتابعات بتلك الموجودة ببنك الجينات عرفت هذه السلالات بـ

Cronobacter muytjensii GH10
Achromobacter xylosoxidans GH9OP
Pseudomonas aeruginosa GHNO8
Pseudomonas putida GH4SNO/P

كانت قدرة هذه السلالات فى تكسير الديازينون كالتالى وبترتيب السلالات 92.59%, 97.75%, 91.82%, and 90.78% مقارنة بتكسيره بنسبة 16.99 فى البيئة بدون بكتيريا.

أما فى حالة الكلوربيريفوس فكان نسبة تحلله بهذه السلالات كالتالى وبنفس ترتيب السلالات 93.43%, 78.51%, 93.18% and 95.36% مقارنة بنسبة تحلل 4.28% فى البيئة التى لا تحتوى بكتيريا وذلك بعد عشرين يوما من التحضين حيث استخدم الديازينون بتركيز 600 مللى جرام للتر والكلوربيريفوس بتركيز 480 مللى جرام للتر.

الكلمات الدالة: المركبات العضوية الفسفورية، التحلل البيولوجى، جين 16s rDNA، التحليل الكروماتوجرافى

تحكيم: ا.د فتوح الدمياطى
ا.د محمد سراج الدين عبد الصبور