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Biodegradation of Organophosphorus Pesticide (Malathion) by *Bacillus* sp. FYM31 Isolated from Agriculture Drainage Water

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Abstract:

Organophosphorus pesticides (OP) are used extensively in many arenas including agriculture and industry leading to humans and agroecosystems disorders. Malathion is one of the OP that are used in agriculture to control pest and protect crops. Also, they harm non-target organisms and affect cruelly water sources, air, and soil quality. The present study aimed to isolate and identify a potent bacterial isolate capable of degrading malathion. Bacterial strain that isolated from Al Fayoum governorate, Egypt exhibited high efficiency for malathion biodegradation. Biodegradation process using minimal salt medium (MSM) supplemented with different malathion concentrations indicated that the bacterium was able to degrade and use malathion as a sole carbon source up to 700 mg/l at 37°C.The potent strain that exhibited biodegradation potential was identified as *Bacillus* sp. FYM31 and deposited into GenBank with the accession number OK325597. HPLC proved the effectiveness of malathion removal by *Bacillus* sp. FYM31 after 12 days of incubation to the level of 70.1% malathion (700 mg/l) degradation. Organophosphorus hydrolase (*opd*) gene was detected in the potent *Bacillus* sp. FYM31 strain. Due to the widespread usage of malathion in Egypt's agricultural areas, *Bacillus* sp. FYM31 can help bio-remediate the polluted areas.

Keywords: Organophosphorus biodegradation, Malathion degrading- bacteria, *Bacillus* sp. Organophosphorus hydrolase, (*opd*) gene

Introduction

Pesticides are used to increase agriculture productivity and prevent the spread of plant diseases. However, their excessive and inconsiderate usage affects the environment and becomes one of the factors of climate change **[1, 2]**. For pests' control, an organophosphorus insecticide has been extensively applied **[3]**. Malathion is considered one of the first and most

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commonly used OP and is still used extensively in Egypt, especially for agriculture **[4]**. The harmful effect caused by malathion can reach the nervous system, a cholinesterase inhibitor that operates as a non-systemic insecticide and has acute toxicity. Overstimulation of the cholinergic pathways is one of its features **[5]**. Malathion was reclassified as hazard class IIA based on findings of its carcinogenicity in human populations **[6]**. Moreover, malathion application has a risk to human health and safety. Also, it causes agroecosystems deterioration, groundwater contamination and soil degradation due to its residual problems **[7, 8].** Additionally, the target organisms become resistant to the pesticides as a result of excess use **[9]**. Malathion has half-lives of 1 to 25 days in soil and 1.5 days to 21 weeks in water **[10]**. The persistence of malathion residues and the related health hazards make the development of a biological elimination approach for the environment is essential. Bioremediation is one of the most efficient, secure, and financially viable procedures to repair contaminated water and soil with pesticides. Microbial enzymes which can catalyze a variety of reactions, including hydrolysis, oxidation-reduction, desulfurization, dehalogenation, and ring cleavage, can be used in bioremediation to degrade and change intractable pesticides **[11]**. Phosphotriesterase (PTE) was identified as organophosphorus hydrolase (OPH) which is the initial enzyme that can hydrolyze a range of organophosphorus compounds by breaking P–O and P–S bonds **[12]**. OPH enzymes are members of the metallo-dependent hydrolases superfamily and encoded by eight genes sharing similar structure, *opdA*, *opd, opaA*, *pte RO*, *pdeA*, *parC*, *mpd* and *phnE* involved in biodegradation. Organophosphorus degrading genes are categorized by gene conservation analysis to four super families including Metallo-dependent hydrolases, Lactamase B, MPP, and TM PBP2 **[13]**. During their active cellular metabolism, these enzymes can transform insoluble organic phosphorous into soluble and accessible phosphorus **[12]** and degraded by some bacterial strains into sulfons, oxons, or other compounds that may be less hazardous than the initial molecules **[14]**. Several researchers have found wide range of microbial species that can break down malathion, such as *Bacillus thirongiensis, Bacillus cereus, Bacillus licheniformis, Ochrobactrum* sp.*, Pseudomonas stutzeri, Bacillus* sp.*, etc* **[10, 15, 16]**. The aim of this investigation was to isolate and identify potent bacterial isolates that can successfully degrade malathion.

Materials and methods

Chemicals and culture media

Malathion (57%) was obtained in commercial form from Central Agricultural Pesticide Laboratory (CAPL), Agriculture Research Center, Giza, Egypt. Chemicals used in this study were from Sigma, Aldrich, USA.

To study bacterial growth and pesticide degradation, mineral salt medium (MSM) was used. It contains (g/l): $MgSO_4.7H_2O$, 0.2; $FeSO_4.7H_2O$, 0.001; KH_2PO_4, 1.5; Na_2HPO_4, 1.5; $(NH_4)_2SO_4$, 2.0 and $CaCL_2.2H_2O$, 0.01; pH 7.0 **[17].** Luria-Bertani medium (LB) was used for bacterial growth and contains (g/l): yeast extract, 5; tryptone, 10 and sodium chloride, 5; pH 7.0. (TM Media, India).

Sample collection

Water samples were collected in December 2019, from an agricultural water drainage in Itsa city, Al Fayoum Governorate, Egypt. Each sample was taken in sterile bottles and kept at 4°C in an ice box for 2–6 hours before being transported to the lab.

Isolation of malathion degrading bacteria

Five hundred ml of each collected water sample was centrifuged for 10 minutes at 6,000 rpm. The pellet was resuspended in 5 ml sterile distilled water, mixed well and inoculated in a flask containing 100 ml of MSM with 100 mg/l Malathion. Cultures were then incubated in dark (to prevent photo-degradation) shaking incubator at 37ºC and 120 rpm for 15 days. Every 5 days of incubation serial dilutions of the master cultures spread on mineral salt agar plates with 100 mg/l of Malathion pesticide and incubated at 37ºC for 2–3 days. The bacterial isolates were further purified and tested for Malathion biodegradation at higher concentrations **[18]**.

Selection of the most potent bacterial isolate

The pure bacterial cultures were transferred separately to MSMmedia containing different concentrations of Malathion (300, 500, 600 and 700 mg/l) and incubated at 37ºC and 120 rpm for 15 days. After that, bacterial growth was estimated through determination of viable cell count/ml (CFU / ml) **[19, 20]**.

Identification of the selected bacterial isolate

Morphological and biochemical characterization

The most tolerant bacterial isolate (FYM31 isolate) was identified via morphological and biochemical properties according to the standard methods recommended by **[21, 22]**.

Molecular characterization

Using Wizard® Genomic DNA Purification Kit Cat. No. A1120 (Promega, USA), a single colony of FYM31 isolate was purified according to the manufacturer's instructions. FYM31 isolate was identified by16S rDNA sequencing as molecular technique. Polymerase Chain Reaction (PCR) was used to amplify the 16S rDNA region from the extracted DNA of FYM31isolate using an Applied Biosystems 2720 Thermal Cycler. Assay was run in 25 µl volumes containing 5 µl of 10X PCR buffer (Promega), 5µl $MgCl_2$ (25 mM), 0.5 dNTPs mixture (each 2.5 mM), 0.5 µl of each Forward (5′-AGAGTTTGATCCTGGCTCAG-3′) and Reverse (5′-CTACGGCTACCTTGTTACGA-3′) **[23]** and 0.3 Go *Taq* DNA polymerase (5u μl^{-1}), 1 µl template (approximately 20 ng μl^{-1}) and 15.7 µl double distilled water. The following conditions were used for PCR amplification: initial denaturation at 94°C for five minutes followed by 35 cycles of denaturation at 94°C for one minute then, annealing at 65°C for one minute followed by elongation at 72°C for one minute and final extension at 72°C for seven minutes. The amplified products were subjected to electrophoresis analysis on 1.0% agarose gel stained with ethidium bromide (0.5µg ml^{-1}) and were visualized on UV gel documentation system (BioRad, USA). Following the manufacturer's recommendations. PCR products were sequenced by LGC (Applied Biosystems™/ Thermo Fisher Scientific, Germany). DNA sequence was compared to GenBank sequences using BLASTn tool to identify the best matches from GenBank based on percent sequence identity (https://blast.ncbi. nlm.nih.gov/Blast.cgi**) [14, 24, 25]**.

Sequence alignments and phylogenetic analysis

PCR products were purified and sequenced using both primers, then the nucleotide sequences were aligned with other sequences downloaded from MEGA 11 Software **[26].** The aligned sequences were saved as Fasta files that have been used as a matrix to estimate the phylogeny of the entire alignments by constructing Neighbor-Joining (NJ) tree in MEGA 11 Software. The phylogenetic tree was constructed and performed with 100 bootstrap replications to evaluate the reliability of the constructions. The evolutionary distances, which are measured in terms of the number of base changes per site, were calculated using the p-distance method.The phylogenetic tree was constructed from the identified 16S rDNA bacterial sequence with their respective reference sequence from GenBank using MEGA 11 Software **[26].**

CIRCOS_configuration_tool.(http://circos.ca/tutorials/lessons/configuration/distribution_and_installation/) were used for sequence alignment of the different sequences and to illustrate nucleotide regions that have a similarity to each other and sequences length **[27]**.

Biodegradation studies

Inoculum preparation

Overnight grown colonies of FYM31 isolate on MSM plate supplemented with 700 mg/l of malathion were washed twice in sterile saline (0.9% NaCl), Centrifuged at 4°C and 3300 rpm for 10 minutes, then re-suspended in sterile saline. The bacterial suspension's optical density was then adjusted to 1 at 600 nm using Spectronic-21 spectrophotometer and was used as inoculum **[28]**.

Biodegradation of malathion by FYM31 isolate

Biodegradation experiment was carried out in 250 ml flasks containing 95 ml of MSM with 700 mg/l of Malathion. Five ml inoculum was transferred to each flask then, flasks were incubated on a shaker incubator at 37°C and 120 rpm. Samples were taken periodically after 3, 6, 9 and 12 days and centrifuged at 4°C and 3300 rpm for 15 minutes. Cell free supernatants were used for the quantification of residual pesticide by HPLC (Uninoculated flasks were used as control).

Malathion residual analysis using high-performance liquid chromatography (HPLC)

Equal volumes of acetonitrile and culture supernatant were mixed, and then the mixture was shaken on a reciprocal shaker for 30 minutes at 150 rpm **[29].** The supernatant was filtered through a 0.22 µm nylon filter. Then, Waters 2690 Alliance HPLC system with a Waters 996 photodiode array detector was used to analyze the concentrated residues. A C18 Reverse Phase Alltech analytical column (4.6 x250 mm, 5µm) was used and maintained at 30^{o}C in a column oven. A 0.45 µm cellulose filter was used to filter the mobile phase (a mixture of 80% acetonitrile and 20% water) before use. The mobile phase was then allowed to run at a rate of 2 ml min^{-1}. A 100 µl of each sample was manually injected into the HPLC system each time. Peak retention time in samples were compared to those of peaks in the pure analytical standard in order to identify the suspected pesticide **[30].** Data calculation according to with significant coefficient of determination R2 as 0.998, and the equation was y = 2.4788x + 0.7474. Hence, to acquire chromatograms for the examination of standard and test reactions, the validated method was used **[31].**

Degradation percentage of malathion was calculated according to the following equation **[32]**:

$$\textbf{Degradation (\%)} = \frac{\textbf{(Residual amount in control} - \textbf{residual amount in sample)}}{\textbf{(Residual amount in control)}} \times 100$$

Detection of malathion degrading gene in *Bacillus* sp. FYM31 strain

Malathion degrading gene of the selected bacterial strain was detected by the presence of organophosphorus hydrolase encode as *(opd*) gene using specific primers, forward primer (*opd* F169) sequence is 5'CGCGGTCCTATCACAATCTC3' and reverse primer (*opd* R169) sequence is 5'CTTCTAGACCAATCGCACTG3'**[33]** with using1.5 µl of standard 1kb DNA ladder (Thermo Scientific, USA) were loaded in 1% agarose gel. Digital images were obtained for PCR products bands using Bio-Rad's Gel Doc XR+ system. Primers were purchased from Macrogen Oligo Co. (Teheran-ro, Gangnam-gu, Seoul, Korea), PCR and amplification steps were done as mentioned before for 16S rDNA PCR. The sequence results were aligned to GenBank sequences using BLASTx tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Isolation and characterization of malathion degrading bacterium

Among the isolated bacteria from water samples collected from agricultural water drainage in Itsa city, Al Fayoum Governorate, Egypt, one bacterial isolate (FYM3) was tolerant and showed considerable growth, which was expressed as bacterial count (CFU/ml) with different malathion concentrations (100, 300, 500, 600, 700 mg/l) (**Fig. 1**). FYM31 isolate was Gram positive rods, with smooth and milky-white colonies. Its biochemical characteristics were shown in **Table 1**.

Further, molecular characterization of the 16S rRNA gene was done by BLAST and CIRCOS programs. BLAST results revealed that FYM31 isolate displayed 99.98% sequence similarity with *Bacillus* sp. strains. Moreover, CIRCOS and phylogenetic tree analysis results showed that FYM31 isolate was closely placed with *Bacillus* strains (**Fig. 2**). Therefore, based on these data the bacterium was submitted to the GenBank databases as *Bacillus* sp. FYM31 with (Accession No. OK325597).

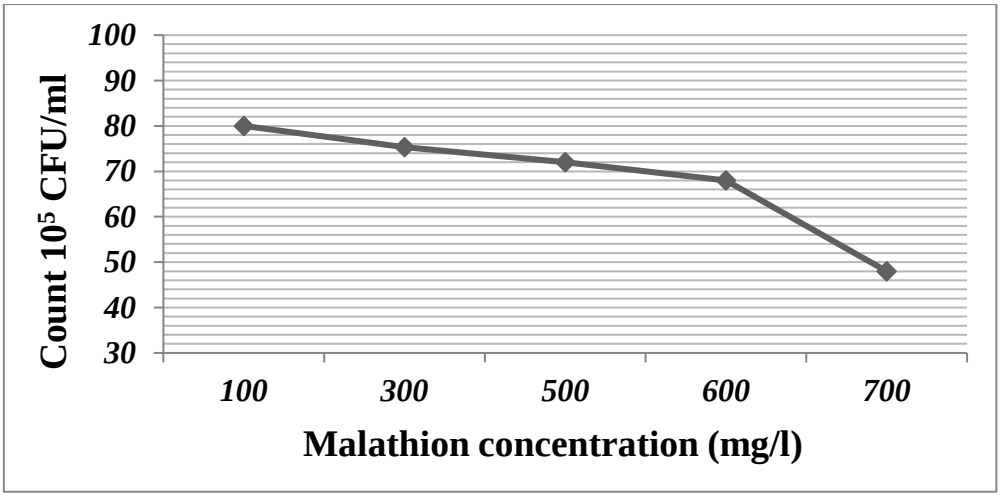


Fig. 1: Viable count of bacterial isolate FYM31 at different concentrations of malathion

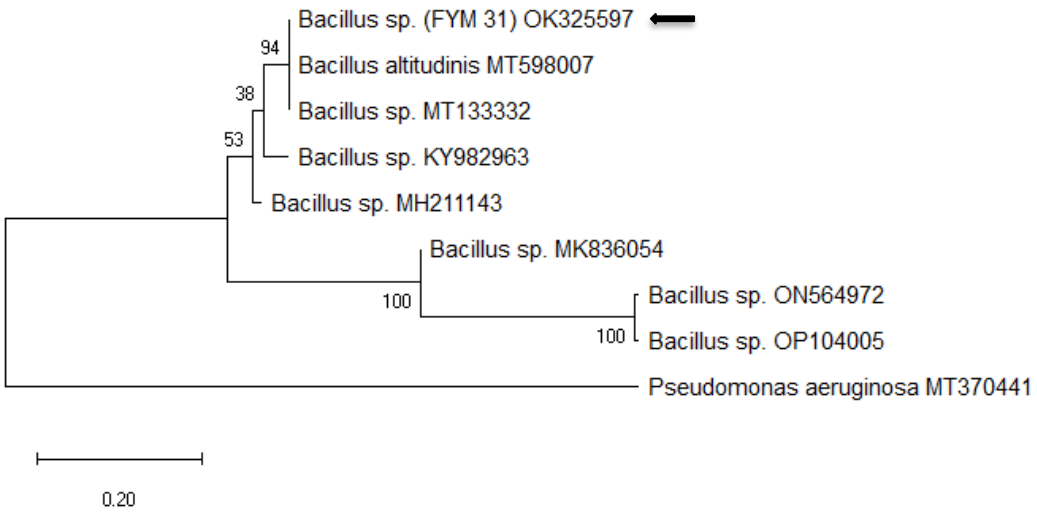


Fig. 2: Phylogenetic tree representing the relationships of16S rDNA gene sequence analysis of *Bacillus* sp. FYM31 as shown by arrow and other closely related strains, numbers at the nodes indicate the percentages of occurrence in 1,000 bootstrapped tree

Table (1): Biochemical characteristics of the bacterial isolate FYM31

Biochemical test	Result
Catalase Test	+
Urease Activity	-
Gelatin Hydrolysis	+
Starch Hydrolysis	-
Indole Production	-
Methyl Red Test	+
Voges-Proskauer Test	+
Citrate Utilization Test	+
Glucose Fermentation	+
Fructose Fermentation	+
Lactose Fermentation	–
Maltose Fermentation	+
Sucrose Fermentation	+

+: Positive result −: Negative result

Malathion degradation by FYM31 strain

The current study revealed that *Bacillus* sp. FYM31degraded malathion (700 mg/l) up to 70.1% (maximum degradation) within 12 days (**Fig. 3**). It is worth noting that degradation rate is increasing gradually and reached more than 66% of degradation within the first nine days of the biodegradation process after which, the rate of biodegradation reduced, and maximum malathion biodegradation (70.1%) required three days. HPLC chromatogram of the extracted control samples' showing malathion peak intensity/area (retention time (RT) of 11.8 minutes) and the extracted tested samples after different incubation time intervals are shown in (**Fig. 4)**. A significantly lower peak area was noticed in samples incubated for 12 days proving that *Bacillus* sp. FYM31 is effective in degrading malathion.

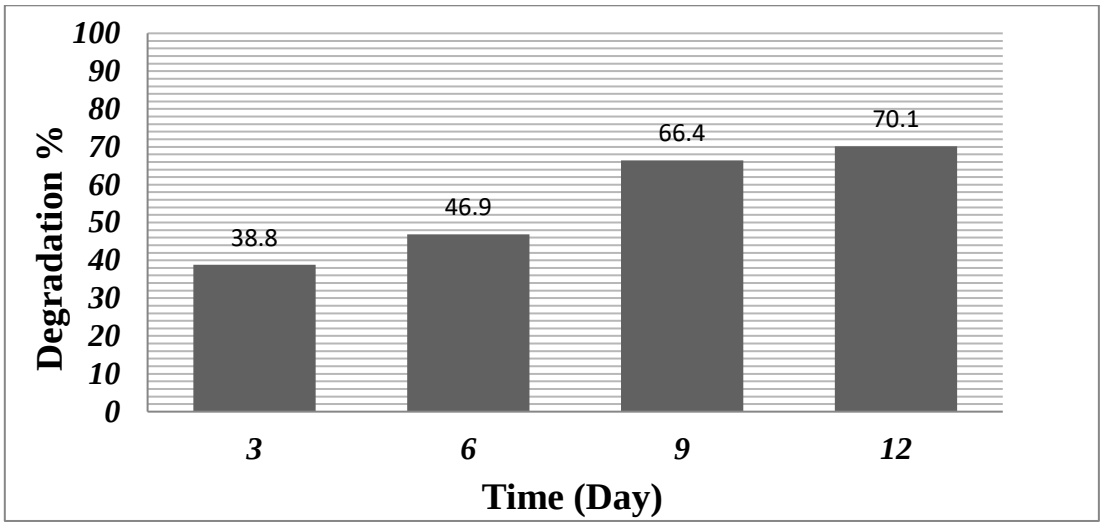
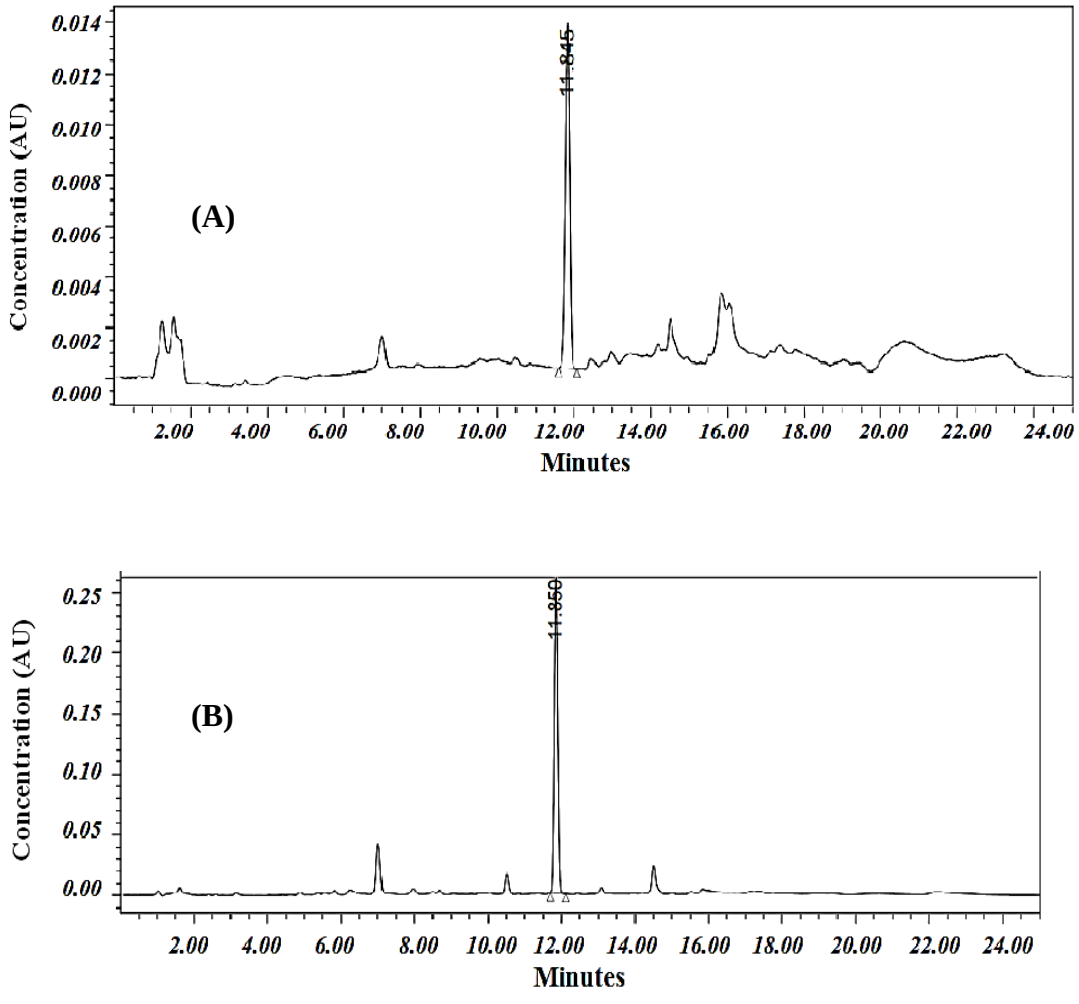


Fig. 3: Degradation percentages of malathion (700 mg /l) by *Bacillus sp.* FYM31



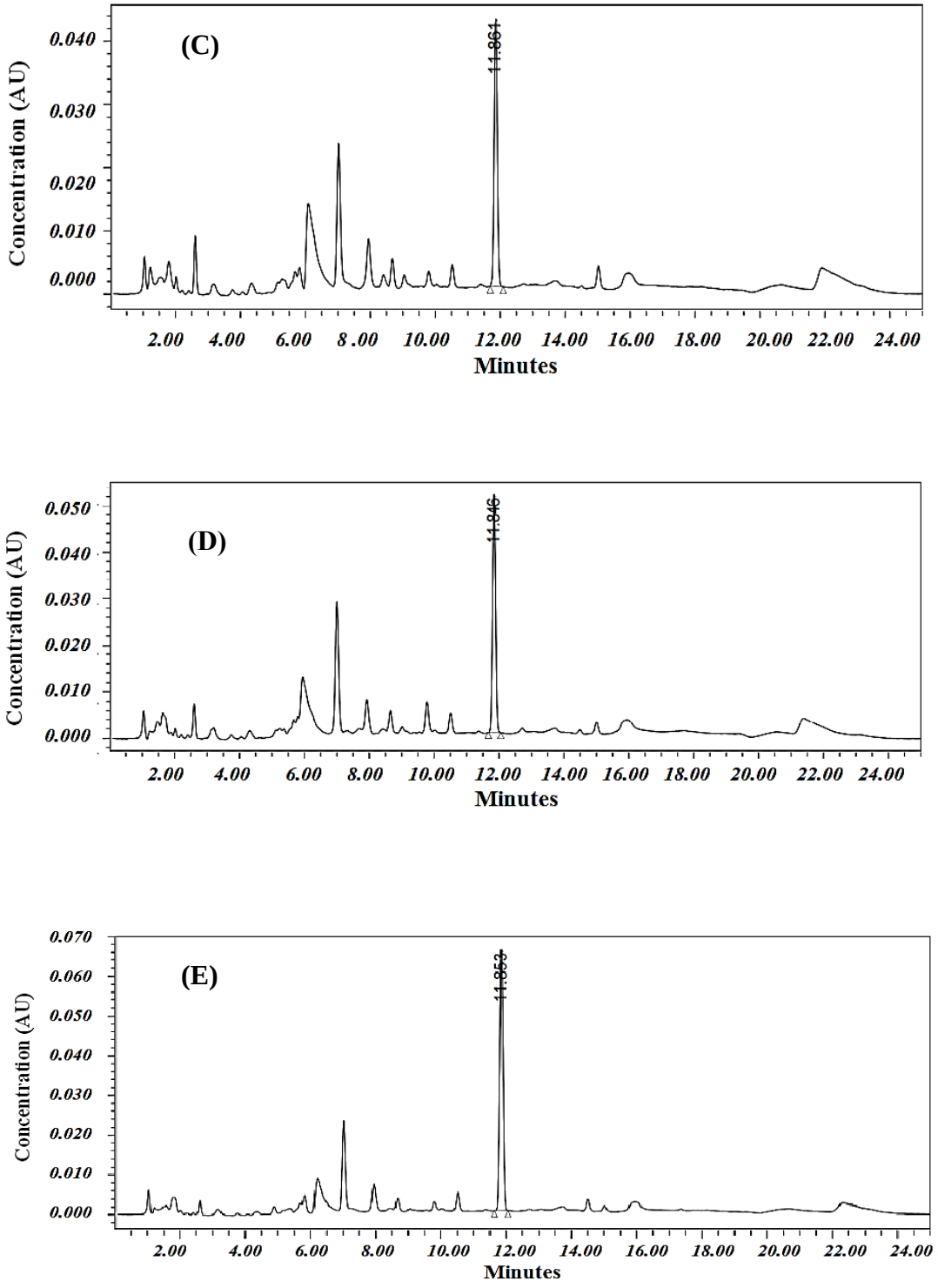


Fig4: HPLC chromatography analysis of malathion degradation % by *Bacillus* sp. FYM31. (A): Malathion standard (atR_T of 11.84 minutes), (B): Degradation%=38.8 after 3days, (c): Degradation% =46.9after 6 days (D): Degradation %=66.4 after 9days and (E): Degradation %=70.1 after 12 days.

Detection of malathion degradation genes in FYM31 strain

The primers *opd* F169 and *opd* R169 efficaciously amplified ~890 bp amplicon size (**Fig. 5).** *opd* gene is sequenced and subjected to analysis on (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). *opd* gene sequence is a member of the Metallo-dependent hydrolases superfamily and are mainly hydrolytic enzymes involved in organophosphates hydrolysis.

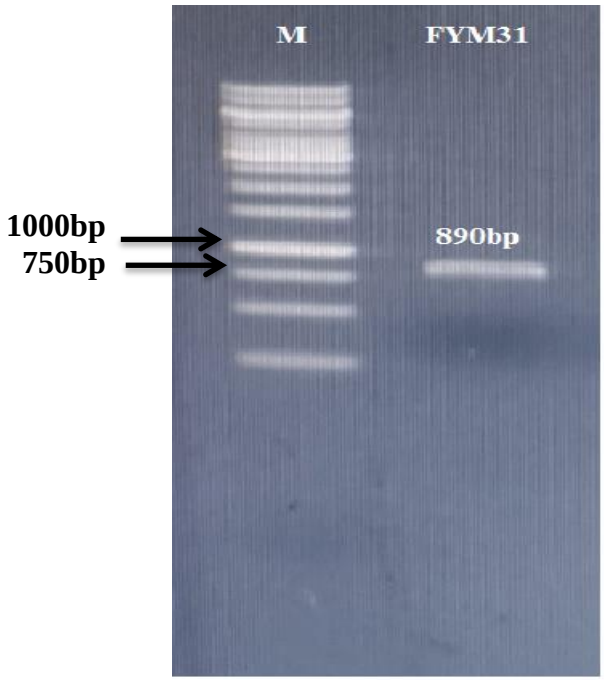


Fig (5): Gel electrophoresis band pattern of amplified products *opd gene,* lane M represents1kb DNA ladder.

Discussion

Organophosphorus pesticides have been broadly applied **[3]**. Malathion is a member of organophosphorus that is widely distributed, persistent, and extensively utilized in Egypt **[34].** The less expensive and less hazardous alternative to more costly and hazardous techniques including chemical and physical procedures is biodegradation **[35]**. It is a process of transforming pesticides into simple, harmless chemicals using the enzymes that are typically generated by bacteria **[36]**.

One of the effective bacterial strains that able to degrade malathion (OP pesticide) was obtained in this study and was morphologically, biochemically and molecularly identified as *Bacillus* sp. FYM31 with accession number OK325597. These results are consistent with earlier researches that demonstrated numerous malathion-degrading bacteria which have been isolated

from a variety of environments, including *Serratia marcescens* **[16]**, *Pseudomonas* **[10, 37, 38]**, *Bacillus* sp. *AGM5* **[29]**, *Pseudidiomarina homiensis* strain FG2 and *Pseudidiomarina* sp. strain CB1 **[39]**.

This study also demonstrated that *Bacillus* sp. *FYM31* able to degrade 70.1% of malathion (700 mg/l) within 12 days. Malathion degradation by other bacterial genera was also reported where the bacteria utilized the pesticide as source of carbon and phosphorous. In this regard, the most common and efficient bacterial genera that have been found are *Bacillus* and *Pseudomonas* **[40]**. Accordingly, several studies identified variety of microbial species that can degrade malathion, including *Acinetobacter johnsonii, Bacillus thirongiensis, Bacillus cereus, Lysinibacillus* sp. *Acinetobacter baumannii, Bacillus licheniformis, Pseudomonas putida, Rhodococcus rhodochrous, Spingomonas* sp., etc. **[14, 15, 41, 42, 43, 44, 45, 46]** also were mentioned for their potential to degrade malathion at a range of concentrations from 5 to 250 mg/l in mesophilic conditions. Among them, *Serratia marcescens* recorded 65% degradation activity within 5 days at malathion concentration 50 mg / l **[18]**, *Pseudomonas stutzeri* was capable of degrading malathion at concentration of 50 to 200 mg /l in a significant manner **[10]**. *Escherichia coli* IES-02 degraded 99% of malathion (50 mg/l) in 4h **[37]**. Complete degradation of 100 mg/l malathion was achieved by *Ochrobactrum* sp. **[38]** and *Bacillus* sp. AGM5 given 72.5% malathion degradation at 300 mg/ml in 15 days **[32].** Meanwhile, the function of the degradation enzymes *in vivo* is significantly defining the strain's capacity for degradation **[47]**. Some studies considered enzymes with known substrate preferences and catalytic properties, used as malathion is the preferred substrate for the proof-of-concept, it should be pointed out that *Opd* is highly effective in degrading a range of OP-based pesticides as noted by **[49]**. Most of the studied bacteria seemed to degrade OP pesticides by the same biochemical pathway with the isolation and characterization of the involved enzymes in this process **[47]**. Organophosphate hydrolyase gene *Opd* was identified in *Enterobacter* sp. **[49]**, *Cronobacter muytjensii, Pseudomonas aeruginosa and Achromobacter xylosoxidans* **[14]**. The functional protein identified was organophosphate pesticide hydrolase and partition protein C. The superfamily was identified for *opd* genes, using BLASTp tool. The gene conservational analysis showed that superfamily of organophasphate hydrolysis gene identified were Metalldependent hydrolases (Metallophosphatase) and may participate in efficient degradation of some organophosphorus compounds **[50, 51, 52]**.

Conclusion

Malathion is the most persistent pesticide residues in the Egyptian agriculture wastewater. *Bacillus* sp. FYM31 has high potential for malathion degradation and was capable of efficiently degrading 70% of 700 mg/l malathion within 12 days. This finding provides valuable method for the bioremediation in malathion contaminated soil and water. Further studies are needed for gene expression and function.

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الملخص العربي

التحلل الحيوي لمبيدات الآفات الفسفورية العضوية بواسطة Bacillus sp. FYM31

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الملخص

تستخدم مبيدات الآفات العضوية الفسفورية على نطاق واسع في العديد من المجالات بما في ذلك الزراعة والصناعة مما يؤدي إلى اضطرابات للبشر والنظم البيئية الزراعية. الملاثيون هو أحد مبيدات الفسفور العضوي المستخدمة في الزراعة لمكافحة الآفات وحماية المحاصيل، ولكنه يضر أيضا بالكائنات غير المستهدفة كما يؤثر بشدة على مصادر المياه والهواء وجودة التربة. الطريقة الاقتصادية، الصديقة للبيئة والناجحة لإزالة التلوث من المناطق التي تضررت من متبقيات مبيدات الآفات هي المعالجة الحيوية باستخدام الميكروبات. هدفت الدراسة الحالية إلى عزل وتعريف عزلة بكتيرية قوية قادرة على تحلل الملاثيون. أظهرت السلالة البكتيرية المعزولة من محافظة الفيوم بمصر كفاءة عالية في التحلل الحيوي للملاثيون حيث استطاعت ان تتحمل وتستخدم كل تركيزات الملاثيون المختبرة (100-700 مليجرام/ لتر) كمصدر وحيد للكربون والطاقة. وقد تم تعريف هذه السلالة البكتيرية على انها *Bacillus* sp. FYM31 وتم ادراجها في بنك الجينات برقم ادخال OK325597. وأثبت تحليل HPLC فاعلية سلالة *Bacillus* sp. FYM31 في تحليل 70.1% من الملاثيون (700 مليجرام/ لتر) بعد 12 يوم من التحضين. كما تم الكشف عن جين هيدرولاز الفوسفورالعضوي في سلالة *Bacillus* sp. FYM31. حيث يستخدم الملاثيون علي نطاق واسع في الأراضي الزراعية بمصر فان استخدام سلالة *Bacillus* sp. FYM31 يمكن ان يساعد في المعالجة الحيوية للمناطق الملوثة.