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Biodegradation of Malathionby *Pseudomonas* Spp. and *Bacillus* Spp. Isolated Frompolluted Sites in Egypt

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Abstract: Malathion is a widely used organophosphate pesticide as it is applied to control a wide range of sucking and chewing pest of field crops, fruits and vegetables. The present study reports the isolation of malathion-degrading bacteria using enrichment technique. Morphological, biochemical and 16S rRNA identification was carried out. The analysis showed that one of the isolated bacteria was belonging to *Bacillus* genusand the other was assigned *Pseudomonas aeruginosa*. The two malathion-degrading isolates were grown in Minimal Salt Medium (MSM) containing different concentration of Malathion (6841 ppm, 14253 ppm, 28506 ppm and 42759 ppm). Malathion residues (degradation products) were measured with Gas Chromatography/Mass Spectrometry after 7 days of incubation at 35°C with the isolated strains in parallel with control samples. Results indicate that *Pseudomonas aeruginosa* and *Bacillus* spp. were able to utilize malathion a sole carbon and energy source even at high concentration and to degrade it co-metabolically. The study revealed that soil bacterial isolates can be used in bioremediation of environmental pollution caused by malathion.

Key words: *Pseudomonas aeruginosa* · *Bacillus subtilis* · Malathion · Biodegradation · Bioremediation · 16S rRNA and sequencing

INTRODUCTION

In modern agriculture practices, farmers apply large number of pesticide for protection of crops from pests and diseases. Pesticides help to increase the agriculture production. Pesticides use is still indispensable in all countries in the area of agriculture also sanitary measures [1].

The World Health Organization has estimated that there are 3,000,000 cases of pesticide poisonings annually, which result in approximately 200,000 deaths. Many of these cases are due to accidental or deliberate intoxication with neurotoxic organophosphate pesticides (Ops) [2].

The term bioremediation has been introduced to describe the process of using biological agents to remove toxic waste from environment [3]. Bioremediation uses biological agents, mainly microorganisms *i.e.* yeast, fungi or bacteria to clean up contaminated soil and water [4].

Bioremediation is the most effective management tool to manage the polluted environment and recover contaminated soil. The hazardous wastes generated from the chemical processes/operations are being treated using physico-chemical and biological methods by the respective industries to meet the prescribed standard as per the Environmental Protection Act, 1986 [3].

Malathion (O,O-dimethyl-S-1, 2-bisethoxycarbonylethylphos-phorodithioate) is a wide-spectrum pesticide of the organophosphate chemical family and is widely used throughout the world. It is one of the earliest organophosphate pesticides introduced in the 1950s and is used widely for agricultural, residential and public health purposes because it enhances food production and provides protection from disease vectors. Malathion is a potential hazard to the environment. Adverse effects induced by malathionin mice, reptiles, birds, earthworms and insects have been investigated [5]. Malathion inhibits acetyl cholinesterase and pseudocholinesterase, resulting

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in accumulation of acetylcholine and overstimulation of acetylcholine receptors in synapses of the nervous system and neuromuscular junctions. In addition, acute exposure to malathion will cause body-wide symptoms and their intensity will be dependent on the severity of exposure. Possible symptoms include nausea, diarrhea, cramps, excessive sweating, seizures and even death [6].

The history of the use of microorganisms by man is as old as the human civilization itself. Microorganisms have long served humans in industrial applications *e.g.* production of food, drug and cosmetics. Recently, progress can be seen in the use of microorganisms for environmental biotechnology, namely removal of greenhouse gases from various sites [7].

However, the aim of the present study was to evaluate the natural biodegradability of *Bacillus subtilis* and *Pseudomonas aeruginosa* to degrade malathion and shed some light on using them to clean up malathion-contaminated soils.

MATERIALS AND METHODS

Sampling and Characterization: Samples were collected from various agricultural fields in different regions of Beni-Suef governorate. Soil samples were collected by removing about 5 cm of the soil surface; 100 g were collected from 10-cm depth and used within 2 h of collection. The soil was moist at the time of collection (moisture level was about 8-9% for the different soil samples). Moisture level was determined by drying the samples in the oven at 110°C and calculating the difference in weight after drying. A commercial grade of malathion (57%) was purchased from the local market.

Isolation of Microorganisms: The enrichment culture technique was used for the isolation of malathion-degrading organisms from different geographical location. The bacterial culture capable of degrading malathion was isolated from the malathion-pretreated soil with varying concentration of malathion in the medium. The soil samples were inoculated into 250 mL of nutrient broth medium in 500-mL Erlenmeyer flask. At daily intervals, one loop full from enrichment culture of the flask was streaked onto nutrient agar plates supplemented also with malathion (0.1-1%) and incubated at 35°C for 48 h. Individual colonies were sub-cultured into nutrient agar plates containing the same concentration of malathion until pure culture was isolated [8].

Identification of the Bacterial Isolates: The identification and characterization of the bacterial culture was made using morphological, biochemical and molecular tests. The morphological analysis comprises shape, motility and Gram staining.

The biochemical tests included catalase test, gelatinasetest, starch hydrolysis test (amylase test), casein hydrolysis test and oxidase test. The molecular tests included 16S rRNA gene detection and identification. 16S rRNA was amplified and sequenced using Applied Biosystem 3130 sequencer. The obtained sequences were BLASTed on NCBI database [9].

Biodegradation Assay: For growth study, 5 mL of 24 h old culture prepared in nutrient broth was inoculated into a 45 mL Minimal Salt Medium (MSM) (5.97g $Na_2HPO_4.12H_2O$, 2.27g KH_2PO_4, 1g NH_4Cl, 0.5g $MgSO_4.7H_2O$, 0.02g $MnSO_4.4H_2O$, 0.01g $CaCl_2.2H_2O$, 0.025g $FeSO_4$), These ingredients were dissolved in 1000 mL distilled water) [8].

Different concentrations of malathion were used in the present study (6841 ppm, 14253 ppm, 28506 ppm and 42759 ppm). The incubation period was 7 days at 35°C. No shaking condition was used to keep the environmental conditions where microorganisms already exposed to. Percentage of residual malathion was determined 7 days post inoculation by Gas Chromatography Mass Spectrometry (GC/MS)[10]. A control experiment without insecticide in MSM was used for comparison.

RESULTS

Soil Isolates: In the present investigation, soil samples were collected from different agriculture soils in Beni-Suef governorate, out of 12 isolates four were found to be *Pseudomonas aeruginosa* and two were found *Bacillus* species by using nutrient agar supplemented with malathion and the rest of the isolates were discarded.

Characterization of the Malathion-Degrading Bacteria: Bacterial characterization, based on the morphological, biochemical and molecular tests indicated that the isolated strains were *Pseudomonas aeruginosa* and *Bacillus subtilis*. Further, the results of the present study indicated that the isolated bacteria were belonging to the *genus Pseudomonas* and they were Gram-negative, rod shaped (Fig. 1), oxidase positive, motile, give positive for catalase test (Fig. 2), positive for gelatinase test (Fig. 3) and was reported to degrade malathion. Whereas, bacteria belonging to the genus *Bacillus* were Gram-positive, large rod shaped (Fig. 4), spore forming, positive for starch hydrolysis test (Fig. 5), positive for gelatinase test and positive for catalase test (Fig. 6).

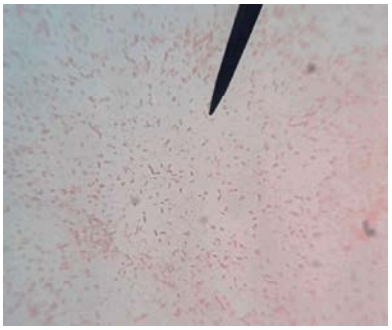

Fig . 1: Gram negative staining of *Pseudomonas aeruginosa*.

Fig. 2: Catalase positive test *of Pseudomonas aeruginosa*.

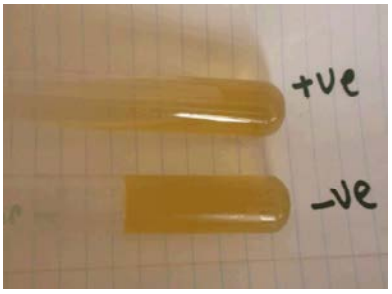

Fig. 3: Gelatinase positive test of *Pseudomonas aeruginosa*.

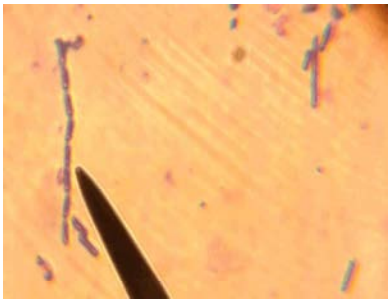

Fig. 4: Gram positive staining of *Bacillus subtilis*.

Biodegradation Byproducts: Malathion degradation products were indicated by GC/MS at 7 days after incubation. Data indicated that malathion was detected as a parent compound at 9.22 min., molecular formula

Fig. 5: Positive starch hydrolysis test of *Bacillus subtilis*.

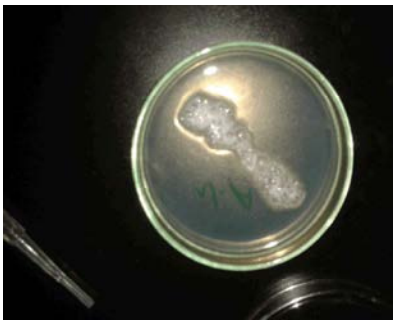

Fig. 6: Positive catalase test of *Bacillus subtilis*.

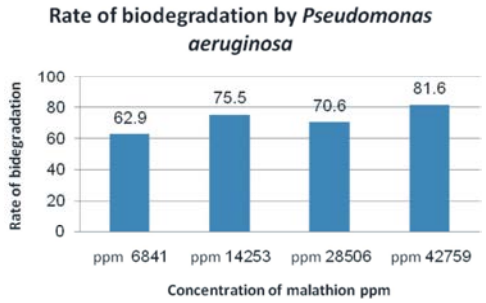

Fig. 7: The rate of malathion biodegrudation performed by *P. aeruginosa*.

Table 1: Rate of degradation of malathion by *Pseudomonas aeruginosa* and *Bacillussubtilis*at different concentration.

Strain/Conc.	6841 ppm	14253 ppm	28506 ppm	42759 ppm
P.aeruginosa	62.9%	75.6%	70.6%	81.6%
B. subtilis	8.5%	82.9%	95.3%	42.6%

$C_{10}H_{19}O_6PS_2$, M. W. of 330, in addition to decrease in malathion concentration ranging from 4% to 57% from the initial concentration.

For the bacterial degradation study, the result of each bacterial degradation (*Pseudomonas aeruginosa* and *Bacillus subtilis*) are represented in Fig. (7), Fig. (8) and Table (1).

Molecular Characterization: After being amplified, 16S rRNA of both *Bacillus* and *Pseudomonas* isolates were

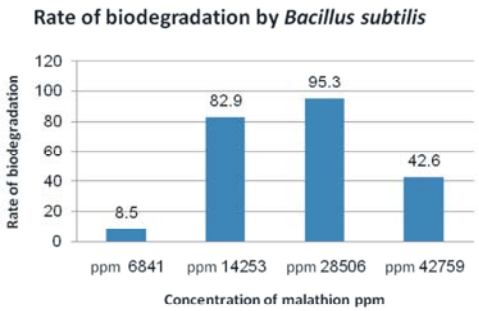

Fig. 8: The rate of malathion biodegrudation performed by *B. Subtilis*

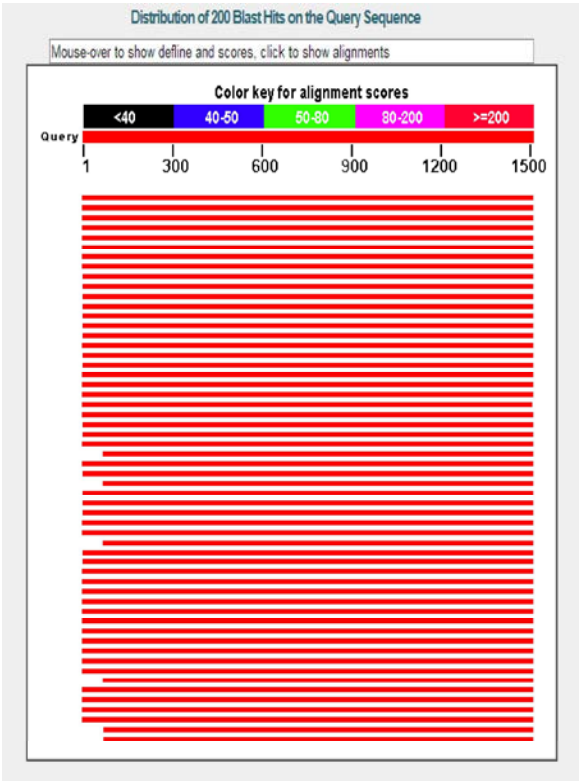

Fig. 9: Graphic representation of the alignment of the sequence obtained from NCBI data base for *Bacillus* isolate.

Fig. 10: The entries produced significant alignments with the target sequence of *Bacillus* isolate.

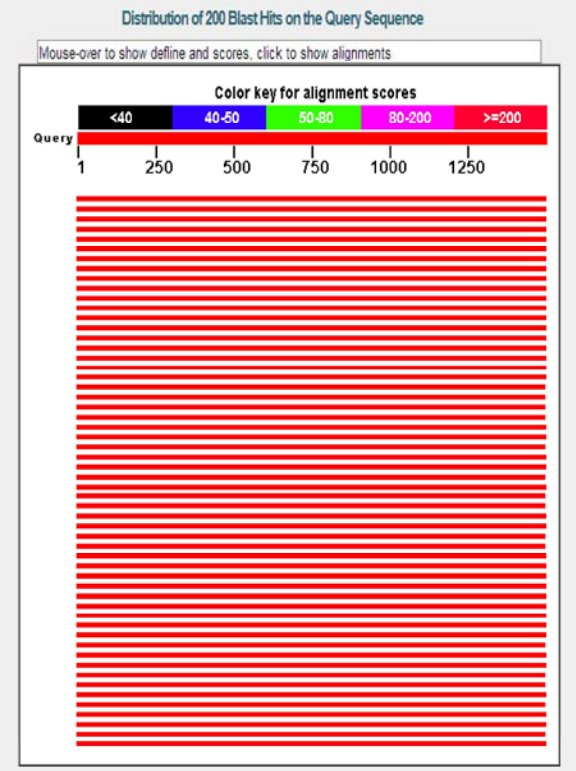

Fig. 11: Graphic representation of the alignment of the sequence obtained from *Pseudomonas* isolate.

Fig. 12: The entries produced significant alignments with the target sequence of *Pseudomonas* isolate.

subjected to sequencing and BLASTed to identify the related sequences in the NCBI database. The obtains data indicated that the nucleotide sequence of the *Bacillus* isolate, when aligned with other sequences, is belonging to *Bacillus subtilis* with 99% relationship with E value = 0 and query coverage = 100% (Figure 9 and 10). While the sequence of *Pseudomonas* isolate was aligned with relationship of 99% with *Pseudomonas aeruginosa*with (E value = 0 and query coverage = 100%) (Figure 11 and 12).

DISCUSSION

Pesticides could increase agriculture productivity which side by side result in the environmental pollution [11].

In the present study, 12 bacterial isolates were isolated on MSM with malathion as a carbon and energy source. On the basis of morphological, biochemical and molecular characterization, *Pseudomonas* and *Bacillus* species were identified [12] and based on the molecular

identification using 16S rRNA sequencing, the two isolates were classified as *Pseudomonas aeruginosa* and *Bacillus subtilis*.

In the present study, we found that *Pseudomonas aeruginosa* was capable to degrade malathion at different concentration (6841 ppm, 14253 ppm, 28506 ppm and 42759 ppm) with biodegradation rates were 62.9, 75.6, 70.6 and 81.6%, respectively. These data were in accordance with many researchers [13-15].

For the first concentration (6841 ppm), the biodegradation rate by which *Pseudomonas* degraded malathion was 62.9%. This might be slightly high rate of biodegradation performed by the genus *Pseudomonas* [16] and it could be attributed to the bacterial enzymes which were secreted with the bacterial cells upon being exposed to the pollutant [8, 17].

In the second concentration (14253 ppm), the biodegradation rate was 75.6%. It was obvious that the rate of biodegradation was increased with increasing the malathion concentration, as it was flow within the hypothesis postulating that the bacterial enzymes concentration was proportionally produced with increasing concentration of malathion [18].

In the last 2 concentrations; 28506 ppm and 42759 ppm, the rate of biodegradation has increased exponentially from 71.6 to 81.6%, respectively with increasing the malathion concentrations applied. This pattern could be considered a linear relationship between the pollutant concentration and rate by which *Pseudomonas aeruginosa* performed the biodegradation process. This relationship might be referred to the readiness of the bacterial enzyme system to degrade malathion extracellularly by secreting the enzymes outside the bacterial cell [19, 20].

Again, at very high concentration (42759 ppm), the degradation rate reached the maximum level where it could be interpreted in the light of adaptability of the bacteria to the surrounding conditions. Tolerance to high pesticide concentrations is critical, since concentrations at contaminated sites may be several orders of magnitude higher than the recommended usage doses for these products [21].

Detoxification of several organophosphates (malathion and parathion insecticides) in the environment was carried out by carboxy esterase and these enzymes are found in many bacteria such as *Pseudomonas aeruginosa* [22].

On the other hand, *Bacillus subtilis* was found to degrade malathionat different concentration (6841 ppm, 14253 ppm, 28506 ppm and 42759 ppm), with rates 8.5, 82.9, 95.3 and 42.6%, respectively. [23, 24], reported similar rate of malathion biodegradation using *Bacillus* sp.

The first concentration was 6841 ppm and the *Bacillus* cultures gave a rate of biodegradation of 8.5% with many different biodegradation products and other impurities. Despite this rate of biodegradation was too low compared to the higher concentrations and compared to the rate of biodegradation performed by *Pseudomonas*, it is still acceptable with respect to the ability of *Bacillus* to cope of a chocking presence of malathion in the media [25, 26].

The second concentration was 14253 ppm. The rate by which *Bacillus* degraded malathion was 82.9%. There was a witnessed increase in the biodegradation rate and this might be related to the ability of the bacteria to cope with this concentration. Other scholars suggested that the higher concentrations of malathion might induce the expression of bacterial enzyme that hydrolyzes malathion [5 19, 25].

The third concentration was 28506 ppm and the rate of biodegradation by which *Bacillus* degraded malathion was 95.3%. It was noticed that the rate of biodegradation has increased with increasing the concentration and that might be referred to as a sort of reactivation of the enzymes related to the biodegradation process. It has been documented [19] that the higher concentration of malathion might have severe effect of the growth curve of *Bacillus* spp., but the pattern of biodegradation obtained in the present study indicated that the microbe was able to cope with the high concentration of malathion.

The fourth concentration was 42759 ppm and the rate of biodegradation presented by *Bacillus* was 42.6%. Results indicated that the highest concentration applied in the present study had adverse effects of the bacterial growth. This concentration might inhibit the bacterial enzymes involved in the biodegradation process. Other researchers indicated a similar pattern of bacterial response to high concentration of pesticide [8, 17, 23].

Data indicated that the biodegradation rate has decreased with increasing malathion after certain concentrations and this might be due to the harsh and stressful conditions the culture exposed to [15].

Another reason may be the less availability of dissolved oxygen (DO), as it is reported that increased organic load might decrease the DO concentration [27].

The obtained results concludes that the bacterial strains isolated from agriculture soil, classified as *Pseudomonas aeruginosa* and *Bacillus subtilis*, could

be useful for the treatment of malathion-contaminated soils and detoxification of agriculture waste even at high concentration.

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