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Biodegradation of Malathion by *Acinetobacter baumannii* Strain AFA Isolated from Domestic Sewage in Egypt

Ahmed F. Azmy, Amal E. Saafan, Tamer M. Essam, Magdy A. Amin, Shaban H. Ahmed

***Abstract*—**Bacterial strains capable of degradation of malathion from the domestic sewage were isolated by an enrichment culture technique. Three bacterial strains were screened and identified as *Acinetobacter baumannii* (AFA), *Pseudomonas aeruginosa* (PS1), and *Pseudomonas mendocina* (PS2) based on morphological, biochemical identification and 16S rRNA sequence analysis. *Acinetobacter baumannii* AFA was the most efficient malathion degrading bacterium, so used for further biodegradation study. AFA was able to grow in mineral salt medium (MSM) supplemented with malathion (100 mg/l) as a sole carbon source, and within 14 days, 84% of the initial dose was degraded by the isolate measured by high performance liquid chromatography. Strain AFA could also degrade other organophosphorus compounds including diazinon, chlorpyrifos and fenitrothion. The effect of different culture conditions on the degradation of malathion like inoculum density, other carbon or nitrogen sources, temperature and shaking were examined. Degradation of malathion and bacterial cell growth were accelerated when culture media were supplemented with yeast extract, glucose and citrate. The optimum conditions for malathion degradation by strain AFA were; an inoculum density of 1.5x 10^{12}CFU/ml at 30°C with shaking. A specific polymerase chain reaction primers were designed manually using multiple sequence alignment of the corresponding carboxylesterase enzymes of *Acinetobacter* species. Sequencing result of amplified PCR product and phylogenetic analysis showed low degree of homology with the other carboxylesterase enzymes of *Acinetobacter* strains, so we suggested that this enzyme is a novel esterase enzyme. Isolated bacterial strains may have potential role for use in bioremediation of malathion contaminated.

Keywords*—Acinetobacter baumannii*, biodegradation, Malathion, organophosphate pesticides.

I. Introduction

ORGANOPHOSPHORUS pesticides (OPPs) are considered to be the oldest and the most widely used pesticides for agricultural practice and animal protection as well as for public health, accounting for an estimated 34% of world-wide insecticide sales [1], [2]. Severe environmental pollution has been caused by widespread use of OPPs since their applications in agricultural areas do not remain at their target sites, but often can enter aquatic environments also [3], [4]. The toxic action of OPPs is caused due to inhibition of acetyl cholinesterase in an irreversible manner leading to increase of the acetylcholine concentration at the synaptic junction resulting in a continuous stimulation of the muscle or nerve fiber and may cause spasms, incoordination, convulsions, paralysis and ultimately death [5].

The general structure of most OPPs is similar, containing three phosphoester bonds. Organophosphorus compounds have a stable and covalent carbon to phosphorus (C-P) bond that is resistant to chemical hydrolysis, thermal decomposition and photolysis [6]. Many authors indicated that the bacterial strains belonging to the different taxonomic groups have a great degradation potential of the organophosphorus insecticides and other pesticides [7], [8]. Studies on microbial capabilities of degradation are important in the development of new bioremediation strategies for the detoxification of different pesticides [9]. Bioremediation has been received great attention because of its reliability and cost-effective to clean up polluted environments [10].

Malathion, S-(1,2-dicarbethoxyethyl)-O,O-dimethyldi-thiophosphate, is an organophosphate insecticide that has been used for some time as a DDT substitute for the control of field crop pests, household insects, flies and animal parasites [11], [12]. It has been known as the first organophosphorous insecticide with high selective toxicity [13]. But still health and environmental hazards of malathion have led to an increasing need to its residual detoxification [5]. Egypt is considered to be an agriculture based country, therefore OPPs including malathion are largely used for public health and agricultural purposes. Soil contamination by malathion can also lead to surface water and groundwater contamination. Some researches on malathion biodegradation had been carried out in Egypt [12], [14]-[16].

Hydrolysis of organophosphate compounds is the most significant step in their detoxification as the compounds will be more vulnerable to further degradation. Esterase or phosphotriesterase enzymes are responsible for catalyzing this reaction [4]. A wide range of microorganisms possessing the organophosphate hydrolase enzyme had been found by research works [6], [17]. Carboxylesterases, a group of relatively nonspecific enzymes, are widely distributed in nature and catalyze the hydrolysis of carboxylic acid esters to their respective acid anion and alcohols. Some carboxylesterases that had been characterized were purified from different vertebrates and plants [18], [19].

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Carboxylesterases can degrade OPPs, carbamates, pyrethoid insecticides, and organic chloride pesticides [20], [21]. Usage of significant quantities of these enzymes for the removal of OPPs in waste water treatment had been proposed by several researchers [3], [22]-[25].

The aim of the present study was to isolate, identify and characterize to the molecular level a bacterial strain capable of degrading some of organophosphorus pesticides particularly malathion and factors affecting its biodegradation. Molecular characterization of the degrading enzyme was also aimed.

II. Materials and Methods

A. Chemicals and Media

Malathion (MAL) analytical grade was purchased from MP Biomedicals (LLC, USA), Diazinon (DIZ), fenitrothion (FEN) and chlorpyrifos (CPF) were of technical grade and supplied by Central Agricultural Pesticide Laboratory, Ministry of Agriculture, Egypt HPLC-grade acetonitrile and water were purchased from LAB-Scan (Poland). All other chemicals were of analytical grade.

A mineral salt medium (MSM) (pH 7.0 ± 0.1) contained (g /ldeionized water), K_2HPO_4 2; KH_2PO_4 1; $NaNO_3$ 4; $CaCl_2$ 0.1; KCl 1; NaCl 1; $MgSO_4.7H_2O$ 0.2 and 1 ml of Focht trace elements solution [26]. Focht trace elements solution contains (mg/l) $MnSO_4.H_2O$ 169; $ZnSO_4.7H_2O$ 288; $CuSO_4.5H_2O$ 250; $NiSO_4.6H_2O$ 26; $CoSO_4$ 28 and $NaMoO_4.2H_2O$ 24 (pH 7.2 ± 0.2). Luria-Bertani (LB) medium composition was (g/L deionized water): Tryptone 10.0; yeast extract 5.0 and NaCl 10.0 (pH 7.0 ± 0.2). The above liquid media were solidified by adding 2% noble agar (w/v) (Difco,USA).

B. Samples Collection and Isolation of Malathion Degrading Bacteria

Twenty-three and thirteen agriculture wastewater and domestic sewage samples, respectively, were collected from May 2011 to May 2012, from different sites from Beni-Suef City, Egypt. Nearly 50-100 ml of each sample was collected in 250 ml sterile conical flasks that were stored at 4°C till use.

Organophosphorus compounds were dissolved in methanol and sterilized by membrane filtration using a pore size of 0.22 µm. MSM was autoclaved, cooled to 40°C and then supplemented with the required concentration of the organophophorus compound. Ten milliliter of each sample was added to 90 ml of MSM supplied with MAL at a concentration of 20 mg/l as a sole source of carbon and energy, incubated at 30°C in a rotary shaker (REMI, India) at 150 rpm for 14 days. After 3 successive transfers, the enrichment culture was serially diluted and spread on MSM plates containing 100 mg/l of MAL. After 3 d of incubation at 30°C, bacterial isolates were selected. Discrete colonies based on morphological properties were selected and subcultured to obtain pure culture.

C. Characterization of Bacterial Isolates

Selected isolates were biochemically identified according to [27]. Identification was confirmed by sequencing of partially amplified 16S rRNA gene of the bacterial isolate.

Molecular characterization of the selected strain was carried out according to modified [28] and [29]. Briefly, genomic DNA was extracted with GenElute Bacterial Genomic DNA Extraction Kit (Sigma-Aldrich, St Louis.MO, USA) according to manufacturer instructions. The 16S rRNA gene was amplified using the universal primer pair: 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) (Invitrogen, USA) and 1525r (5′-AAGGAGGTGWTCCARCC-3′) (Invitrogen, USA) producing an amplicon of approximately 1500 base pair (bp). Polymerase chain reaction (PCR) was carried out using Dream Taq Green PCR Master Mix (2X) (Thermo Fisher Scientific Inc., USA) in a total volume of 50 µl, containing 10-20 ng of template DNA and 100 pmol of each primer. Thermo cycling conditions consist of a first denaturation for 4 minutes at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 5 min using Primus 25 Advanced (thermo cycler). The amplification was verified by gel electrophoresis using 1% (w/v) agarose gel supplemented with 0.2 µg/ml of Ethidium bromide. PCR products were purified by Gene Jet Gel Extraction Kit (Thermo Fisher Scientific Inc., USA) according to manufacturer protocol. The purified product was sequenced using Bigdye terminator v3.1 cycle sequencing kit. Nucleotide sequence similarities were determined using other known sequences found in the GenBank database using BLAST program of National Center for Biotechnology Information (NCBI) databases and neighbor-joining phylogenetic trees were constructed with CLUSTALW (http://www.ebi.ac.uk) and tree explorer programs.

D. Inoculum Preparation for Biodegradation Study

Inoculum size of highly MAL degrader was prepared as described by [30]. Briefly, a single pure colony from the strain was grown in nutrient broth medium, harvested by centrifugation at 4600×g for 5 min, washed with autoclaved normal saline and resuspended in normal saline to set an OD590 nm of 0.7. Colony forming units per milliliter (CFU/ ml) of this suspension was quantified by the dilution plate count technique and required inoculums were prepared by adding appropriate amount of normal saline.

E. Extraction of Samples (Pesticide Residues) for HPLC Analysis

One hundred milliliter of MSM supplemented with 100 mg/l of MAL was inoculated with bacterial cells to obtain a concentration of 1.5 $\times 10^8$CFU/ml and incubated at 30°C in orbital shaker at 150 rpm. Malathion residues were detected at different time intervals 0, 1, 2, 4, 7, 11 and 14 d from inoculation. Each treatment was performed in three replicates. A set of medium without microorganism amended with an identical amount of MAL was run simultaneously under identical conditions.

Five milliliters aliquot from the previously mentioned different media were centrifuged at 7200 ×g for 10 min to remove the bacterial pellets, and extracted 2 times with 50 ml of dichloromethane by shaking vigorously. Dichloromethane

extract was passed through anhydrous Na_2SO_3 for dehydration, and then evaporated to dryness using rotary evaporator at 30°C. The dry residue was dissolved in 1 ml acetonitrile and subjected to HPLC analysis. Twenty microliter was injected in HPLC (Agilent Technologies 1260 Infinity, USA), using Eclipse plus C18 (4.6 ×250mm 5 µm) and diode array detector (DAD) at λ 210 nm. HPLC working conditions were acetonitrile: water (70:30), at a flow rate 1 ml/min. Degradation of other organophosphates was also investigated with identical conditions. Flasks with 100 ml MSM medium were supplemented separately with 100µg/ml of DIZ, CPF and FEN. The same absorbance was used for DIZ but for CPF and FEN pesticides the absorbance (λ) wave lengths were 280 nm and 254 nm, respectively, and the concentration of pesticide was determined by HPLC. For DIZ, flasks were kept from the light to avoid photo-degradation.

F. Factors Affecting Malathion Biodegradation

If not otherwise mentioned, all flasks of the different experiments contained 100 ml of the MSM at a concentration of 100 mg/l of MAL, inoculated with 1.5 $\times 10^8$ CFU/ml of bacteria, and then incubated on a rotary shaker at 150 rpm and 30°C with previously mentioned time intervals and the MAL concentration was measured by HPLC. A set e of each composition without inoculation was kept as controls. Triplicate analyses were conducted and mean values were adopted.

Three different carbon sources (glucose, sodium citrate and phenol) and also yeast extract as a nitrogen source were added separately, at a concentration of 1 g/l to the culture media. Parallel to measurement of MAL biodegradation, growth curve of the AFA strain in the presence of different carbon sources was also measured. Aliquots of 3 ml of bacterial growth culture were withdrawn at different time intervals and monitored by measuring the OD600 with a spectrophotometer (Shimadzu, Japan).

To investigate the effect of temperature on biodegradation process, three flasks were incubated at three different temperatures; 25°C, 30°C and 37°C.

The effect of inoculum density was investigated using three different inoculum densities 1.5×10^4, 1.5×10^8 and 1.5×10^{12} CFU/ ml.

To determine the effect of agitation on MAL biodegradation, two sets were applied, a set of flasks incubated on a rotary shaker and another unshakable one.

G. Sequence Analysis of Carboxylesterase Gene

Carboxylesterase enzyme might be responsible for biodegradation of organophosphate pesticides in *Acinetobacter* sp., so a specific primer was designed based on multiple sequence alignment of carboxylesterase enzymes in different *Acinetobacter* sp., The forward primer (5/- CGN CAN GTY TAD TTY GTY -3/) and reverse primer (5/- RTT NCC RTA NGT NGG NSW -3/) (Invitrogen, USA). Primers were analyzed with multiple primer analyzer tool supplied by Thermo Fisher Scientific (USA).

Reactions were carried out using Dream Taq Green PCR Master Mix (2X) (Thermo Fisher Scientific Inc., USA) in a total volume of 50 µl, containing 10-20 ng of template DNA and 100 pmol of primer. Thermo cycling conditions consisted of a first denaturation for 4 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 5 min producing an amplicon ~ 500 bp using Primus 25 Advanced (thermo cycler).

The amplification was verified by gel electrophoresis using 1% (w/v) agarose gel supplemented with 0.2 µg/ml Ethidium bromide, PCR product was purified by Gene Jet Gel Extraction Kit (Thermo Fisher Scientific Inc., USA) according to manufacturer protocol. The purified product was sequenced using Bigdye terminator v3.1 cycle sequencing kit. The BLAST program of NCBI database was used for deduced amino acid identity search. BLASTP was used for the deduced amino acid identity search and BLASTN for the nucleotide sequence identity search (http://www.ncbi.nlm.nih.gov/BLAST)

H. Phylogenetic Analysis of Carboxylesterase

Sequence of 16S rRNA gene of the isolate was aligned with their homologous sequences using Clustal W2 with default settings. Phylogeny was analyzed with MEGA version 6.06 software and distances were calculated using Kimura 2-parameter distance model, clustering was performed with neighbor-joining method.

For carboxylesterase enzyme, ExPASY translate tool was used to convert sequenced nucleotide to their corresponding amino acid sequence. The translated amino acid sequence was aligned with their homologous sequences using Clustal W2 with default settings. Phylogeny was analyzed with MEGA version 6.06 software and distances were calculated using Poisson model, clustering was performed with neighbor-joining method.

I. Statistical analysis

Data were analyzed statistically by one way ANOVA test except shaking factor was analyzed by unpaired T-student test using SPSS software program (SPSS Inc., Chicago, IL, USA), p value <0.05 was considered significant.

III. Results

A. Identification and Characterization of Malathion Degrading Bacteria

Thirty six domestic sewage and agriculture waste water samples collected from different sites in Beni-Suef City, Beni-Suef Governorate, Egypt, were involved in this study for the isolation of organophosphate-degrading microorganisms. Samples were subcultured on liquid medium of MSM supplied with 20 mg/l of MAL, and then on MSM plates with increase concentration to 100 mg/l. Three different bacterial isolates only showed different abilities for degradation of high concentration of MAL. Biochemical patterns supported the identification of the isolates as *Acinetobacter* sp. and *Pseudomonassp.* (Table I).

TABLE I
MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF MALATHION DEGRADING BACTERIAL ISOLATES

Characteristics	AFA	PA	PM
gram stain	Gram negative	Gram negative	Gram negative
shape under microscope	short rods	rods	rods
biochemical tests			
catalase	+	+	+
oxidase	-	+	+
nitrate reduction	-	+	+
esculin hydrolysis	-	+	+
gelatin liquefaction	-	+	-
citrate utilization	+	+	+
lysine decarboxylase	-	-	-
growth at 37°C	+	+	+
growth at 41°C	-	+	-

AFA: *Acinetobacter baumannii* PA: *Pseudomonas aeruginosa*
PM: *Pseudomonas mendocina*.
+: Positive -: negative result

Partial sequencing of 16S rRNA fragment was deposited in the GenBank database. Multiple alignments revealed that isolate number 1 (named PS1) showed high similarity (100%) to *Pseudomonas aeruginosa* strain DBT1BNH3 (Accession Number FJ976651.1). The isolate number 2 (named PS2) show high similarity (99%) to *Pseudomonas mendocina* strain SM5 (Accession Number JX102498.1). The last strain (named AFA) was belonged to genus *Acinetobacter* and show highest similarity (97%) to *Acinetobacter baumannii* strain AC29 (Accession Number CP007535.1). To identify the phylogeny of strain AFA, strains from different species were chosen to construct the phylogenetic tree based on 16S rRNA sequences (Fig. 1).

Acinetobacter baumannii (AFA) strain was selected for detailed organophosphorus degradation studies under various conditions due to its relatively higher degrading efficiency amongst the other isolates. To our knowledge, strain *A. baumannii,* AFA, was the first one of this genus that possessed the ability to metabolize different organophosphorus compounds. For further analyses, the other 2 isolates were reserved.

B. Assay of Different Organophosphates Bacterial Biodegradation

Biodegradation assay of the four organophosphorus pesticides (MAL, DIZ, CPF and FEN) by inoculation of *A. baumannii* strain AFA at a concentration of 100 mg/l in MSM as a sole carbon source for 14 d was carried out. Organophosphorus pesticides degradation was monitored by HPLC. The results showed that, there was no significant biodegradation for 24 h after inoculation, but the isolate degraded MAL, DIZ and FEN with a different efficiency, and after 14 d of incubation 84.8%, 64.2% and 51.9% of the initial dose of the insecticides were degraded, respectively (Fig. 2). On the other hand, AFA strain was able to degrade only 29.7% of initial CPF concentration. In contrast, a non-inoculated control showed no change in MAL organophosphorus concentration for 14 d of incubation.

C. Effect of Different Carbon Sources on Bacterial Malathion Degradation

Growth of bacterial isolates and biodegradation rates of MAL by *A. baumannii* AFA strain were checked with different carbon and nitrogen sources (glucose, sodium citrate, phenol and yeast) along with MAL. The OD measurements at 600 nm showed a steady increase in bacterial mass. The growth of bacteria was the most effective with yeast extract followed by glucose and then citrate within about 7 d of incubation. The least rate of growth was observed with phenol and MAL with no significant difference between them (Fig. 3). Simultaneously, the HPLC analysis showed a substantial reduction in the level of MAL. The isolate was capable of degrading MAL in the presence of other carbon sources greater than MAL consumption alone. After 24 hour of bacterial inoculation into 100 mg/l liquid MSM, glucose and citrate significantly increased MAL degradation, with complete MAL disappearance within 14 d as shown in Fig. 4 (a). Addition of yeast extract greatly enhanced the biodegradation ability of AFA strain by starting immediately after bacterial inoculation into culture medium and after 48 h, approximately 75% of MAL was degraded and complete removal of MAL within 7 d.

Malathion degradation by the isolated strain was monitored at temperatures 25°C, 30°C, 37°C, of MSM supplemented with 100 µg/ml of MAL as a sole carbon source. There was non-significant difference between biodegradation at the different tested temperatures till the 7th day. Then the most efficient biodegradation was observed at 30°C, while 37°C was considered the least favorable temperature for the process as shown in Fig. 4 (b). Inoculation of MSM containing 100 µg/mlof MAL with different bacterial concentrations (1.5×10^4, 1.5×10^8 and $1.5x10^{12}$ CFU/ ml) of *A. baumannii* AFA strain was studied. Malathion was degraded by the isolate with the three initial inoculum densities tested. Fig. 4 (c) represents that a lag period of nearly 48 h, 24 h and 2h were needed to start MAL degradation with 1.5×10^4, 1.5×10^8 and 1.5 $x10^{12}$ CFU/ ml, respectively. After 4 days, almost all bacterial inoculums exhibited the same degradation rate, and more than 85% of MAL was degraded within 14d.

The results as presented by Fig. 4 (d), indicated that MAL remaining after 14 d from inoculation of *A. baumannii* AFA into MSM containing 100 mg/l of MAL on a rotator shaker at a speed of 150 rpm compared to another stagnant one was; 15.2% and 43.5%, respectively, of the initial concentration. It was significant difference and also evident the prominent difference in biodegradation rate. In both conditions MAL biodegradation started nearly after 24 h, then degradation rate in shaking condition was steeply, while it was gradually degraded without shaking.

D. Characterization of Carboxylesetrase

PCR primers based on consensus amino acid homology between different carboxylesterase genes (Fig. 5) were designed. The forward and reverse primers successfully amplify a PCR product of 521 bp. ExPASY translate tool of the sequenced nucleotides give partial protein sequence of 158

amino acids (Fig. 6). Although carboxylesterase enzyme of *A. baumannii* strain AFA showed a high similarity to several carboxylesterase, alpha/beta hydrolase, esterase and peptidase enzymes of different Acinetobacter strains, it seems to be phylogenetically distant from the previously described above enzymes. BLASTP analysis indicates that the 158 amino acid sequences had identity of 94% to both carboxylesterase enzyme of *A. baumannii* (WP_015451658) and esterase enzyme of *A.baumannii*(WP_011860269), 93% identity to esterase enzyme of *A. nosocomialis* (WP_004711227) and 89% identity to esterase enzyme of *A. pittii* (WP_017399656) as shown in Fig. 7. We suggested that this enzyme is a novel esterase enzyme, which differs from those previously reported esterase enzymes.

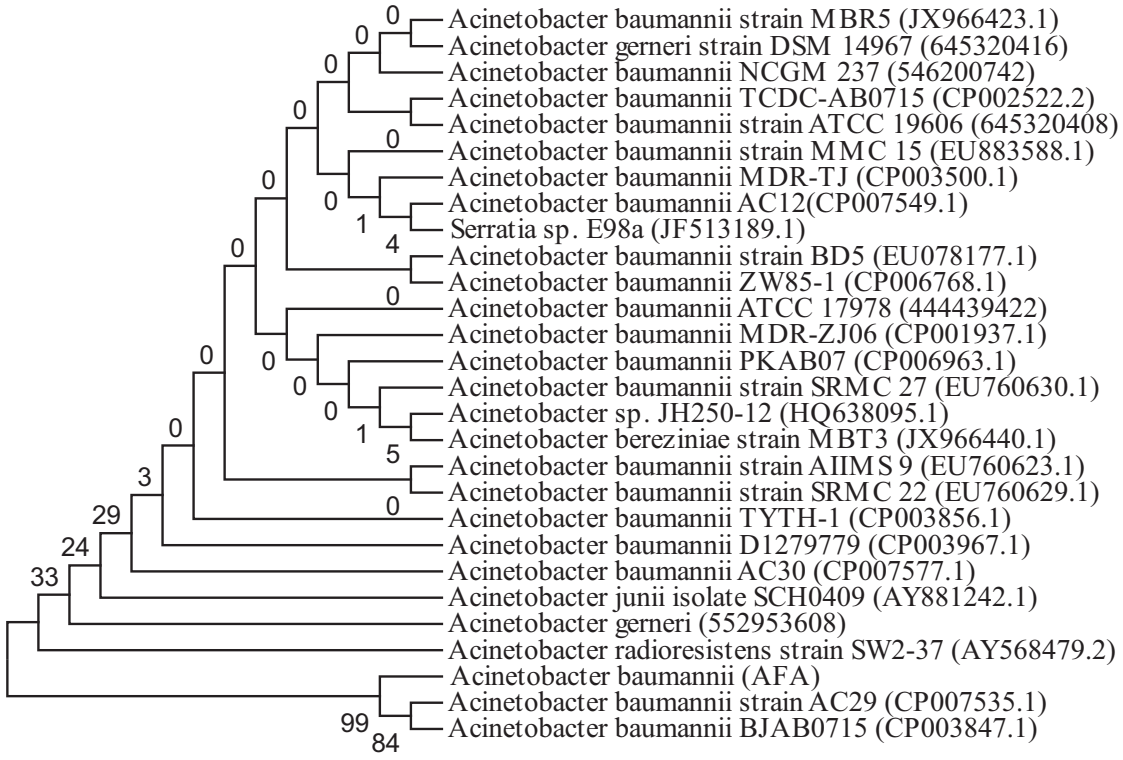

Fig. 1 Phylogenetic tree of *Acinetobacter baumannii* strain AFA based on 16s rRNA gene sequence analysis. Bootstrap values obtained with 500 replications were indicated as percentage at all branches. Gene bank accession numbers are given in parentheses

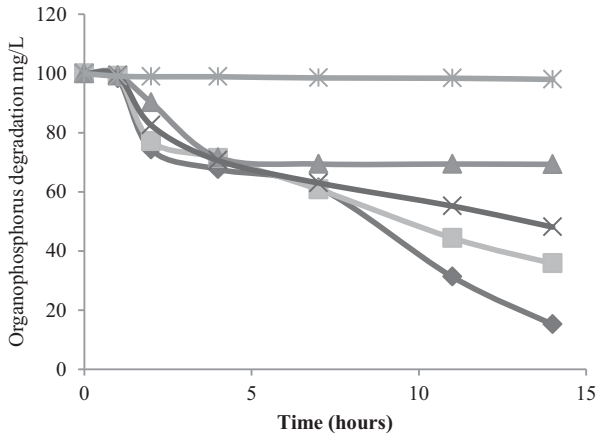

Fig. 2 Biodegradation of different organophosphorus by *Acinetobacter baumannii* strain AFA in MSM containing malathion (♦), diazinon (■),fenitrothion (×) and Chlorpyrifos (▲) at a concentration of 100 mg /l in MSM using each of them as the sole carbon and energy source, and uninoculated (control) (x)

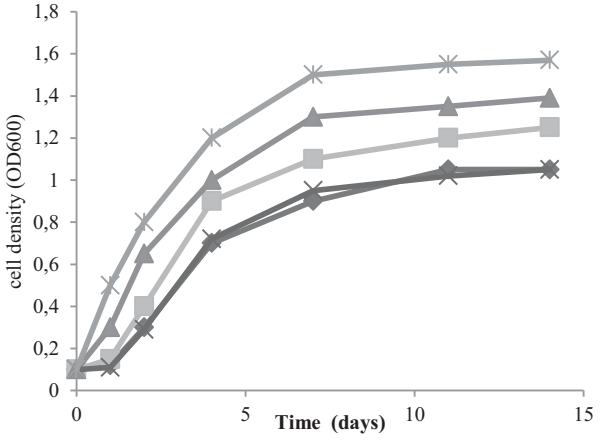

Fig. 3 Microbial growth (cell density) of *Acinetobacter baumannii* strain AFA during malathion biodegradation using it as a sole carbon source (♦) and in presence of different carbon sources as phenol (×), citrate (■), glucose (▲) and yeast extract (X)

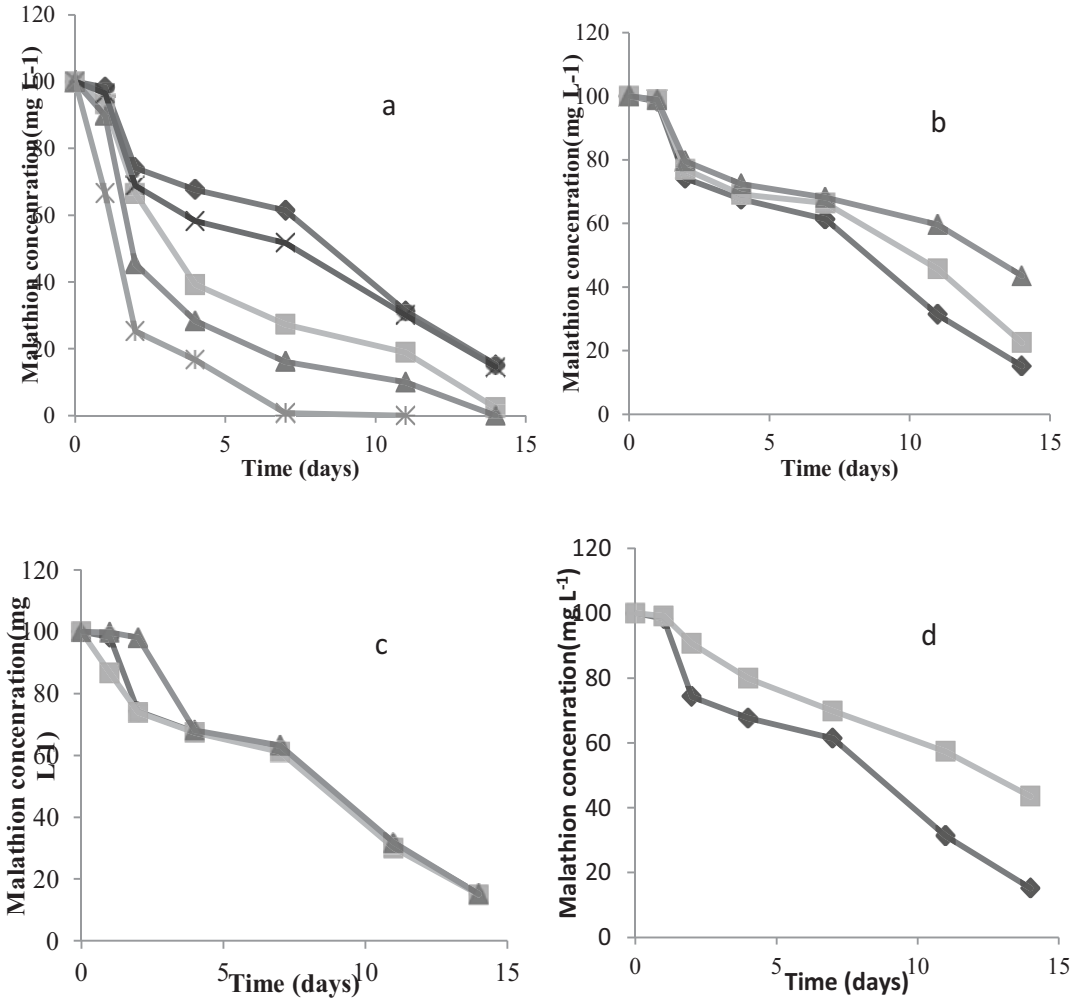

Fig. 4 Effect of different factors on malathion biodegradation by *Acinetobacter baumannii* strain AFA in MSM medium containing 100 mg/l of malathion: (a) a sole carbon and energy source malathion alone (♦), phenol (×) ,malathion + citrate (■), malathion + glucose (▲), and malathion + yeast extract (ɤ); (b) malathion as a sole carbon source at 37°C (▲), at 25°C (■), and at 30°C (♦); (c) malathion with various bacterial inoculum densities; 1.5×10^{4}CFU/ ml(▲), 1.5×10^{8}CFU ml(♦), and 1.5×10^{12}CFU/ ml(■); and (d) malathion as a sole carbon and energy source on rotatory shaker at a speed of 150 rpm (♦) and in stationary case (■)

IV. Discussion

Extensive use of organophosphorus compounds in agricultural makes it is one of the most important and widespread pollutant in our environment. The biological method for detoxification is preferable than physical and chemical methods due to its advantages of low operational costs, low investment, and also environmental friendliness. Some publications have been reported on the malathion biodegradation under aerobic conditions [11], [12], [25], [31]-[33].

The results of plating of different domestic and agriculture waste water samples on MSM agar supplemented with high concentration (100 mg/l) of MAL was three MAL-degrading strains. They were identified by biochemical analysis to the genus level as *Acinetobacter* sp. and *Pseudomonas* sp. Phylogenetic analysis allowed identifying the strains as *Ps. aeruginosa*, *Ps. mendocina* and *A. baumannii* (named *A. baumannii* AFA). *Acinetobacter baumannii* AFA strain in the present study was isolated from a domestic sewage. It was able to grow on high MAL concentration reflecting their ability to use it as a sole carbon, and energy source.

It has been reported that several bacterial genera such as; *Xanthomonas* and *Acinetobacter* [34], *Serratia* and *Pseudomonas* [9], *Flavobacterium* and *Sphingomonas* [35] and *Agrobacterium* sp. [7] may participate in efficient degradation of some organophosphorus compounds. Most of the studied bacteria seemed to degrade OPPs by the same biochemical pathway with isolation and characterization of the involved enzymes in this process [36]. Organophosphorus hydrolase is encoded by the *opd* gene located on plasmids [7], [37]. However, homologous of this gene had been also reported on chromosome [17]. Isolation of catabolic plasmids harbouring *opd* from geographically different regions and taxonomically different species may be necessary in the evolution and widespread of OPPs-degrading bacteria [38], [39]. *Pseudomonas* sp. known as very metabolically active

bacteria and able to utilize many agrochemicals were isolated from various soils contaminated with some OPPs, such as; chlorpyrifos, parathion, methyl-parathion [7], [40]. On the other hand *Acinetobacter* sp. appears to be a new bacterium genus that may participate in efficient degradation of some OPPs.

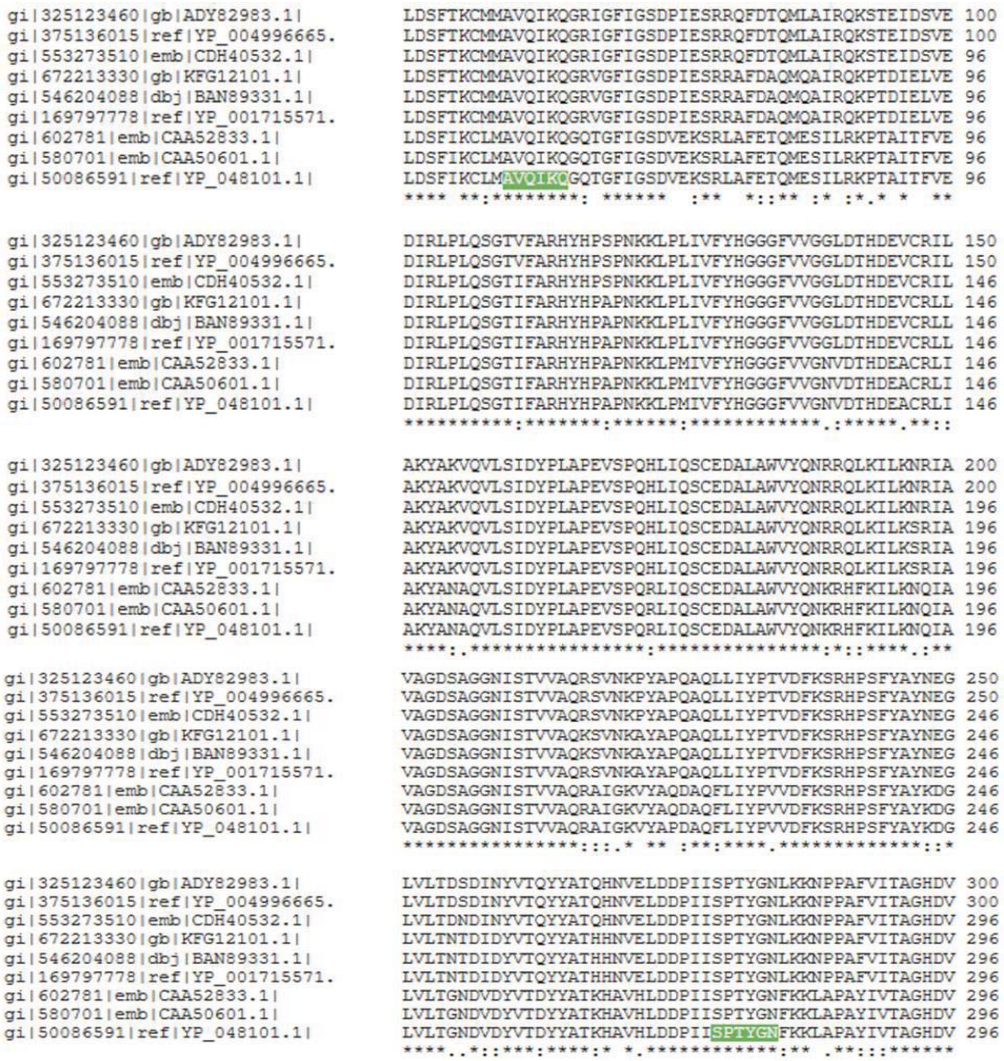

Fig. 5 Multiple sequence alignment of amino acids sequences of carboxylesterase genes from different *Acinetobacter* strains by ClustalW tool, highlighted region show amino acid sequences used in primer design

The aim of selection of *A. baumannii* AFA was due to two reasons. First, widespread of *acinetobacter* in nature and can be obtained from soil, water, living organisms, and even from human skin and secondly, it's high efficient degradation activity as presented from our results. Environmental and biotechnological applications of Acinetobacter have been increased. Many studies reported that *Acinetobacter* sp. can use a wide range of toxicants as biphenyl and chlorinated biphenyl as a sole carbon and energy source [41].

REQLLIYHTVDFKSRHPSFYAYNEGLVLTNTDIDYVTQYYATHHNVELDDPIISPTYGNL

KKNPPAFVITKGACLTSNISVKTKRRKKKRRNKGEFPQTPEPATAILLFNIF

NCLRFTQAIFMNARLNMLWNFRYKDSRLSTGTLPYFAYPTPFSLNL

Fig. 6 Deduced amino acid sequence of carboxylesterase enzyme of *Acinetobacter baumannii* AFA, ExPASY translate tool of sequenced nucleotides

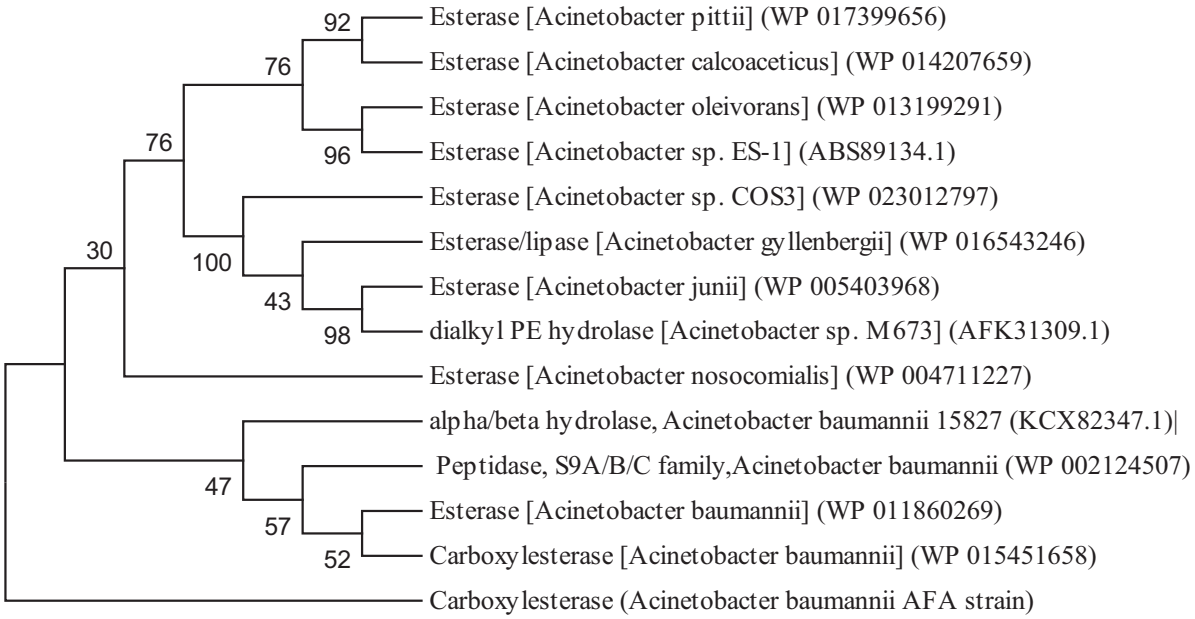

Fig. 7 Phylogenetic analysis of carboxylesterase enzyme and their homology proteins from various bacterial genomes using neighbor-joining method. The bootstrap values obtained with 500 replications were indicated as percentage at all branches. Gene bank accession numbers are given in parenthesis

The isolated bacterium in the present study *A. baumannii* strain AFA had the ability to degrade the four tested OPPs (malathion, diazinon, fenitrothion and chlorpyrifos) that may be derived from similar molecular structure. Their biodegradations were of different degrees starting after about 24h of inoculation. Within 14 d,84.8% of MAL were degraded by the selected strain. As previously mentioned, five bacterial strains were isolated, *Pseudomonas* sp., *Ps. putida*, *Micrococcus lylae*, *Ps. aureofaciens*, and *Acetobacter liquefaciens*, from Egyptian soil that were able to use MAL as a sole source of carbon [12]. While, in another study, about 40.92% and 54.67% of MAL initially added to the medium were degraded by *Brevibacillus* sp. and *Bacillus cereus*, respectively, after incubation for 7 d and no depletion of MAL was noticed after that [42]. This might be due to MAL toxicity and substrate bioavailability.

Diazinon and fenitrothion were degraded beside MAL but to a different extent (64.2% and 51.9%, respectively) that may be due to their difference in chemical complexity and their side chain [43]. Chlorpyrifos was slowly degraded achieving 29.7% removal only in our study. That might be attributed to the production of 3,5,6-trichloro-2-pyridinol (TCP) (one of the main chlorpyrifos metabolites) which have a toxic effect on bacteria that prevented the proliferation of chlorpyrifos-degrading microorganisms [12], [23], [44]. Although both *Stenotrophomonas* sp. YC-1 [29] and *Sphingomonas* sp. strain Dsp-2 [44] and *Alcaligenes faecalis* strain DSP3 [45] degraded 100% of 100 mg/l of chlorpyrifos within 24 h and 12 d, respectively. *Synechocystis* sp. strain PUPCCC 64 degraded 93.8% of 5 mg/l chlorpyrifos within 5 d [13]. *Bacillus pumilus* C2A1 degraded 89% of 1000 mg/l chlorpyrifos within 15 d [31]. This may be explained by high degradation of chlorpyrifos and the capability to degrade TCP.

Some reports showed that the isolated bacterium can utilize organophosphates as a source of carbon or phosphorus [12], [46] from the hydrolysis products [47]. Microbes from different environment that not exposed to certain chemicals have the ability to degrade them by producing highly stable enzymes [48]. This explains detection of organisms capable of utilizing organophosphate compounds from other places with no history of pesticide application [49].

During degradation studies of other researchers at high chlorpyrifos concentrations, they observed longer lag phase with increasing concentration of pesticide. It was proposed that longer lag phase at higher concentration might be because greater number of bacteria were required to start rapid degradation of pesticide. Before rapid degradation occurs, biodegradation possibly starts slowly and requires an acclimation period [50].

According to previous studies on malathion biodegradation, environmental factors such as physical and chemical characteristics of the substrate, nutrients status, co-substrates effects, pH, temperature and biotic factors such inoculum density influence the rate and extent of degradation [51], [52]. Therefore, studies on such factors are necessary if bioremediation will be required.

With our isolate *A. baumannii* strain AFA, using enrichment culture techniques with one of four kinds of additional compounds; sodium citrate, glucose, phenol and yeast were tested to study the effect of another carbon source in malathion degradation. The results showed that yeast, glucose and citrate could promote malathion biodegradation and cell growth, but phenol had no significant effect probably due to its hard nature as another carbon source. In previously mentioned researches that found glucose enhanced the degradation rate of *Bacillus pumilus* isolate and increased the

growth rate of both *Revibacillus* sp. and *Bacillus cereus* strains by 10^5 fold, respectively, as compared to malathion alone [30], [42]. However, different results were reported, they found that the presence of another carbon source, initially it stopped degrading organophosphorus compounds but with the passage of time it degraded it efficiently [2], [4], [12]. The explanation was when glucose was depleted, the bacterium started to utilize organophosphorus compound as a source of carbon, giving bacterium the environmental adaptation. These results did not agree with those from previous studies in which the presence of glucose had no effect on the degrading ability of their bacterial isolates on different OPPs [50], [53], [54]. Other studies demonstrated that the presence of short-chain fatty acids (e.g. acetate or succinate) accelerated cell growth of acinetobacter, reduced the toxicity of the pesticide, and drastically promoted the degradation of malathion, whereas simple carbohydrates (e.g. glucose or fructose) did not have such effect [53], [54]. On the other hand, other findings that addition of glucose or succinate to *Stenotrophomonas* strain had no effect on the degradation pattern of chlorpyrifos [29].

Co-metabolismis defined as the metabolism of an organic compound in the presence of a growth substrate that is used as the primary carbon and energy source [34]. Co-metabolism was suggested by previous reports concerning the isolation of organophosphorus degrading microorganisms [17], [55]-[57]. Two effects of the growth substrate on co-metabolic process: first, it may enhance cell growth and thus stimulate non-growth substrate biotransformation [58]; Secondly, it may act as a co-substrate in pollutant metabolism through induction of certain enzymatic pathways that both the growth substrate and the non-growth substrate may share [56], [58]. The potential expression of organophosphorus degrading enzymes in *acinetobacter* even in the presence of readily available carbon sources, suggested that it should be a stable trait [59]. It is likely that detoxifying enzymes responsible for biodegradation process were almost coded by chromosomal genes.

Temperature is highly variable in different areas, so it is considered as an important factor in malathion biodegradation. *Acinetobacter baumannii* was able to grow over a wide range of temperature (25^{o}C, 30^{o}C, and 37^{o}C). Thirty degree Celsius was the optimum temperature for biodegradation process, while 37^{o}C was the least favorable temperature for the process. The same results were obtained by [29] and [43]. Another study also, showed that culture expressing organophosphorus degrading enzyme constitutively decreased at 37^{o}C but survived at 30^{o}C and lower temperature [60].

Microorganism inoculum size had been identified as a possible cause for the failure or success of biodegradation process upon inoculation with a strain able to degrade the pesticide [51]. In the present study, inversely proportional relationship had been found between the initial inoculums density (1.5×10^4CFU/ ml, 1.5×10^8CFU/ ml, and 1.5×10^{12}CFU/ml) and the length of acclimation period (48 h, 24 h, and 2 h, respectively) and all bacterial inoculums exhibited more than 85% of MAL degradation within 14 d. Similar correlation between the length of acclimation period and inoculum density was observed by [30] and [52]. It had been suggested that at high inoculum density there was higher concentration of bacterial cells versus toxic molecules ratio that would have been in favor of bacterial cells [49]. While, at low inoculum density there was a longer lag phase that might be required for the multiplication of a small active population to reach a certain level which was sufficient to rapidly degrade malathion [30], [61]. Inoculum concentration of 10^6–10^8cells/g soil was recommendable for the decontamination of pesticide-contaminated sites [52]. However in a similar study [62] that found that inoculum size of an *Agrobacterium* strain as low as 10^5cells/g was adequate to degrade atrazine rapidly.

In our study, at a speed of 150 rpm of bacterial culture of AFA strain with 100 mg/l of MAL, compared to another stagnant culture medium; 84.8% and 56.5%, respectively, of the initial MAL concentration were degraded. It had been reported that shaking accelerated the biodegradation process as it made the organism in contact for a longer time with the organophosphorus compound and it had a positive effect on microbial growth increasing their biomass.

Biodegradation is an important environment biotechnology for the treatment of organic pollutants. One important strategy is the use of some enzymes to break down OPPs residues. Monoacid and diacid derivatives are detoxified products of malathion degradation by carboxylesterase enzymes [63].

The finding in our study was that sequencing result of amplified PCR product and phylogenetic analysis indicated that 94% identity to both carboxylesterase enzyme of *A. baumannii* (WP_015451658) and esterase enzyme of *A. baumannii* (WP_011860269), 93% identity to esterase enzyme of *A. nosocomialis* (WP_ 004711227) and 89% identity to esterase enzyme of *A. pittii* (WP_017399656). To our knowledge, the characterized carboxylesterase enzyme in the present study was a novel esterase enzyme.

Studies on microbial carboxylesterase genes had been cloned and expressed as well [64]. [65], but studies on the use of microbial carboxylesterases for pesticide degradation are limited. Sequencing technologies enable high-throughput functional genomic research will be advanced in the next-generation.

The application of compounds and xenobiotics could seriously pollute the surrounding environment. Bioremediation is a cost effective method to degrade toxic compounds into non-harmful products. Successful removalof pollutants involving pesticides surrounding us by implanted bacteria (bioaugmentation) had been previously reported for many compounds [39], [66].

V. Conclusion

In the present study, three bacterial strains belonging to both *Acinetobacter* and *Pseudomonas* genera that may participate in efficient degradation of the organophosphorus insecticides malathion were isolated. *A. baumannii* AFA strain was capable of utilizing not only malathion as a sole carbon source but also other organophosphorus compounds such as diazinon, fenitrothion and chlorpyrifos. However, use of pesticide-degrading microbial systems for removal of organophosphorus compounds from the contaminated sites

requires an under-standing of ecological requirements of degrading strains. Addition of co-substrates as glucose, citrate or yeast extract greatly enhances the biodegradation process of malathion. High inoculum density at 30°C with shaking achieved maximal degradation in this batch experiment system. Partial sequencing of carboxylesterase gene showed 89% to 94% identity to carboxylesterase genes of different *Acinetobacter* sp. There is a need for further research on the biochemical and genetic aspects of malathion degradation by the isolated in our studies bacteria.

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