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ORIGINAL PAPER

Screening for and isolation and identification of malathion-degrading bacteria: cloning and sequencing a gene that potentially encodes the malathion-degrading enzyme, carboxylestrase in soil bacteria

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Abstract Five malathion-degrading bacterial strains were enriched and isolated from soil samples collected from different agricultural sites in Cairo, Egypt. Malathion was used as a sole source of carbon (50 mg/l) to enumerate malathion degraders, which were designated as IS1, IS2, IS3, IS4, and IS5. They were identified, based on their morphological and biochemical characteristics, as *Pseudomonas* sp., *Pseudomonas putida*, *Micrococcus lylae*, *Pseudomonas aureofaciens*, and *Acetobacter liquefaciens*, respectively. IS1 and IS2, which showed the highest degrading activity, were selected for further identification by partial sequence analysis of their 16S rRNA genes. The 16S rRNA gene of IS1 shared 99% similarity with that of *Alphaprotoebacterium* BAL 284, while IS2 scored 100% similarity with that of *Pseudomonas putida* 32zhy. Malathion residues almost completely disappeared within 6 days of incubation in IS2 liquid cultures. LC/ESI-MS analysis confirmed the degradation of malathion to malathion monocarboxylic and dicarboxylic acids, which formed as a result of carboxylesterase activity. A carboxylesterase gene (CE) was amplified from the IS2 genome by using specifically designed PCR primers. The sequence analysis showed a significant similarity to a known CE gene in different *Pseudomonas* sp. We report here the isolation of a new malathion-degrading bacteria from soils in Egypt that may be very well adapted to the climatic and environmental conditions of the country. We also report the partial cloning of a new CE gene. Due to their high biodegradation activity, the bacteria isolated from this work merit further study as potential biological agents for the remediation of soil, water, or crops contaminated with the pesticide malathion.

Keywords Malathion · Pesticide degradation · *Pseudomonas* · GC-ECD · *C*arboxylesterase

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Introduction

Malathion, *S*-(1,2-dicarbethoxyethyl)-*O,O*-dimethyldithiophosphate, is an organophosphate insecticide used extensively to control a wide range of chewing and sucking insects. The U.S. Environmental Protection Agency, EPA, has classified malathion as a toxicity class III pesticide and allowed a maximum

amount of 8 parts per million (ppm) of malathion to be present as a residue in specific crops used as foods (U.S. EPA. Office of Pesticide Programs 1988). Technical-grade malathion (the grade that is usually used for agricultural purposes) may contain up to 11 impurities formed during its production and/or storage, some of these impurities, such as isomalathion, have been found to be significantly more toxic than malathion itself or to potentiate the toxicity of malathion (Uygun et al. 2007). The most toxic metabolite of malathion is the oxidation product malaoxon which formed in the air, and which is responsible for the insecticidal activity of the parent compound (WHO 1997). Malathion irreversibly inactivates acetylcholine esterase at various sites resulting in an accumulation and continues action of neurotransmitter acetylcholine at postsynaptic sites and may cause death (Gosselin 1984).

As the demand for agriculture produce increases, so inevitably does the need for pesticides. It is estimated that 4 million ton of pesticides are applied to world crops annually for pest control. Egypt is no exception in its excessive and frequent application of pesticides including malathion. This result in high levels of its residues accumulated on agricultural crops, which poses a potential health hazard to consumers. The contamination of soil by malathion can also result from bulk handling in the farmyard and the rinsing of containers, and accidental release may occasionally lead to the contamination of surface water and groundwater. These health and environmental concerns have led to an increasing need to the detoxification and removal of residual malathion from water and may be contaminated vegetables especially with its heavy use in Egypt.

Microorganisms capable of degrading organophosphate compounds (OPs), including malathion, may be used for bioremediation of these pesticides (Laveglia and Dahm 1977; Chapalamadugu and Chaudhry 1992; Singh and Walker 2006). These microorganisms may play a significant role in breaking down the active molecules (Chapalamadugu and Chaudhry 1992; Singh and Walker 2006) thus detoxifying the environment.

Bioremediation is a potential solution that is considered to be a viable, environment-friendly approach for dissipation of organophosphate molecules and an attractive alternative to other conventional techniques (Saaty and Booth 1994). It can be effectively used to overcome the pollution problems (Galli 1994; Bhadhade et al. 2002). Several bacterial and fungi species have been isolated and characterized that can degrade malathion (Paris et al. 1975; Lewis et al. 1975; Walker 1976; Singh and Seth 1989; Kim et al. 2005).

The carboxylesterase family (EC 3.1.1.1.) comprises a group of esterases hydrolyzing carboxylic ester bonds, such as is present in malathion, with relatively broad substrate specificity. They show a high degree of sequence similarity and they are believed to be involved in the detoxification of many xenobiotics (Jakoby and Ziegler 1990). In many studies the gene encoding carboxylesterase was cloned and the recombinant protein was expressed (Kim et al. 2003; Zhang et al. 2004; Merone et al. 2005; Kakugawa et al. 2007).

Humidity, temperature, pH and composition of soil nutrients play important roles in the efficiency of microorganisms to degrade pesticides. Temperature, as an example is extremely variable in different countries, differing seasonally and also during the day.

The aim of the present study was to isolate from Egyptian soil and characterize new bacteria capable of degrading malathion as a sole source of carbon and to investigate their ability to degrade other OPs. Despite the fact that several malathion degrading bacteria have been isolated as mentioned above, the new isolates from this work may be better suited to the climate and environment conditions in Egypt. We also aimed to partially clone and determine the DNA sequence of the gene encoding a carboxylesterase from one of the newly isolated strains. This enzyme is isolated from bacteria that efficiently degrade malathion and are adapted to the Egyptian environment. This new carboxylesterase therefore, will be cloned in an environmentally friendly host which then will be used in a detoxification of malathion from soils, water and malathion contaminated food.

Materials and methods

Reagents and chemicals

Analytical grade malathion (PESTANAL, Fluka) was purchased from Sigma. All reagents and solvents used in the present study were of analytical grade.

Samples collection for enrichment studies

About three hundred of soil samples were collected from the surface layer (0–15 cm) from thirty different sites of agricultural fields in Egypt. These fields were mainly in two cities, Fayoum and Giza from a clay soil where we managed to isolate the strains understudy. Samples also collected from sandy soil from Wadi Elnatroun, Cairo. These fields were continuously applied with many pesticides especially organophosphates.

Isolation of malathion-degrading bacteria by enrichment technique

All samples were first enriched with commercial malathion (50 ppm) to increase the probability of finding malathion degraders. After thorough mixing, all preparations were incubated at 30°C for 30 days. Further enrichment was then carried out in which 1 g from previously enriched soils was used to inoculate 50 ml autoclaved M9 minimal salt media (MSM) (Sambrook et al. 1989) containing malathion (50 mg/l) as a sole carbon source. A solution of malathion was sterilized by filtration (0.4 μm) and added to autoclaved media after cooling. Cultures were then incubated in an orbital shaker for 3 days at 30°C and 160 rpm. After 3 days, all cultures were then allowed to settle for 1 h and about 1 ml of each supernatant was used to inoculate another 9 ml fresh autoclaved enrichment media prior to incubation for additional 7 days under the same conditions. After three subsequent transfers into the same media (3 weeks), serially dilutions of the cultures were made from 10^{-4} to 10^{-8} and plated onto agar plates (1.5%) containing the same media. Plates were incubated at 30°C until bacterial colonies become visible (84 h). Five selected isolates were purified and maintained pure at 4°C. They were designated from the two initials of the first two authors as IS1, IS2, IS3, IS4 and IS5.

PCR amplification of the 16S r RNA gene of IS1 and IS2 isolates

Strain IS1 and IS2 were further identified and characterized by using standard biochemical methods (George et al. 2005) combined with 16S rRNA gene sequence analysis. Chromosomal DNA was isolated from bacterial cells by incubation in 0.5 ml water at 100°C for 39 s. The 16S rRNA genes of IS1 and IS2 were amplified using the universal primer pair: 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) (sigma) and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′) (Sigma) which is complementary to the 5′ end and 3′ end of the prokaryotic 16S rRNA gene, respectively (Lane 1991), thereby producing an amplicon of 1500 bp. In this reaction, amplification was carried out in 25 μl reactions by using a PCR master mix kit (Promega) according to the manufacturer's instructions using a GeneAmp PCR System 2400 Thermal cycler (Perkin Elmer). The following program was used: 94°C for 4 min as initial denaturation step, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 2 min, with a final extension at 72°C for 10 min. PCR products were purified by Wizard® SV Gel and PCR cleaning up system kit (Promega) following the protocol provided by the supplier and then resolved by electrophoresis on 1% agarose gels. The purified products were sequenced using the same primers with ABI PRISM model 310 genetic analyzer following provider's recommendations. The obtained sequences were analyzed for similarities to other known sequences found in the GenBank database using BLAST program of the NCBI database.

Inoculum preparation for degradation studies

A single pure colony from the IS2 isolate was streaked onto agar plates of MSM containing malathion (50 mg/l) as the sole carbon source. After incubation at 30°C for 48 h the growth was washed by 1 ml sterile MSM, and 0.2 ml aliquots of the suspension were used to inoculate a series of liquid cultures (20 ml). Liquid cultures were incubated at 30°C on an orbital shaker at 160 rpm. At periodic time intervals constant aliquots were withdrawn and used to assess bacterial growth by measuring the optical density at 600 nm until it reached a maximum using a JENWAY 6105 U.V./VIS spectrophotometer. At this point an aliquot (0.2 ml) was again transferred into fresh liquid media (20 ml). This procedure was repeated twice more to maximize the degrading potential of the isolate. The final liquid cultures produced were then used as an inoculum in all studies described below. A number of controls were also run parallel to samples; (1) uninoculated medium,

(2) medium without malathion inoculated with the isolate and (3) uninoculated medium with malathion.

Degradation of malathion in liquid media

The IS2 inoculum was transferred to MSM media (1% v/v) containing malathion (50 mg/l) as a sole carbon source. At the same time other cultures containing glucose (0.05%), as an alternative carbon source, were also inoculated and all cultures were incubated at 30°C on an orbital shaker at 160 rpm. Non-inoculated media were also run in parallel to the other cultures as control.

Samples were taken 0, 2, 4 and 6 days after inoculation and the residual malathion was extracted Malathion was extracted twice (2 × 30 ml) by addition of an equal volume of petroleum ether to each aliquot. After vigorous shaking for 5 min, the organic layer was separated and dehydrated by passing through anhydrous Na_2SO_4. The solvent was then allowed to evaporate completely and residual malathion was redissolved in n-hexane and subjected to gas chromatography (GC) analysis in National Institute for Standards (NIS), Cairo, Egypt.

Determination of residual malathion by gas chromatography analysis

Residual malathion was analyzed using a GC system (Agilent 6890N) equipped with an electron capture detector (GC-ECD), with an HP5 (5% phenylpolymethylsiloxane) (60 m × 250 μm × 0.25 μm thickness) column. The applied temperatures were 280 and 320°C for the injector and detector, respectively. The oven temperature was held at 200°C for 2 min and then increased at a rate of 5°C/min until it reached 260°C, after which time it was returned to 200 C for each new run. The carrier gas (nitrogen) flow rate was 0.8 ml/min. Different volumes (1, 2 and 4 μl) of a standard solution of malathion (0.1 g/ml) were also injected to construct a standard curve.

Identification of the degradation products by LC/ESI-MS analysis

Strain IS2 was allowed to grow in the same media containing malathion (50 mg/l) as a sole carbon source for 48 h and incubated under the same previous conditions. Cells were collected by centrifugation, and an aliquot (1 ml) was then taken from the supernatant and mixed with 1 ml 2-propanol. The mixture was centrifuged at 10,000 rpm for 3 min and, after freezing (−80°C) for 10 min, was centrifuged again (Yoshii et al. 2007). The extracted products were separated using an HPLC system equipped with a C-18 Hyperpurity column (25 cm × 4.6 mm, 5-μm particle size). The LC separation was performed with a total flow rate of 0.2 ml/min, an oven temperature of 40°C, an injection volume of 10 μl and a gradient elution. The gradient elution was carried out with 0.5 M dibutylamine acetate in water (solvent A) and acetonitrile (solvent B) and the conditions were initially 97% A–3% B, holding at 97% A–3% B for 5 min, programming to 20% A–80% B over 20 min, and holding at 20% A–80% B for 10 min (30 min total analysis time). The column equilibration was accomplished using the initial conditions for 10 min prior to the next injection.

Utilization of other organophosphorus pesticides (substrate range)

One bacterial isolate was incubated in M9 minimal media containing 50 mg/l fenitrothion, diazinon, profenofos or chlorpyrifos pesticides as the sole carbon sources. Cultures were incubated under previous conditions; non-inoculated media were also run as controls. The optical density was measured every 24 h.

Carboxylesterase (CE) assays

For esterase activity measurement, the IS2 isolate was allowed to grow in the same medium under the same conditions. Constant aliquots (1 ml) were periodically withdrawn (for 6 days) from each culture (run in triplicate) and centrifuged for 5 min to collect bacterial pellets. Esterase activities were measured in culture filtrate according to a modified method of Ellman's colorimetric method (Ellman et al. 1961). A standard curve of α-naphthol was constructed to calibrate the results.

PCR amplification and sequence analysis of CE gene; the gene might be responsible for malathion degradation

Specific primers pairs (CE-f and CE-r) were manually designed based on the consensus amino acid sequence

from different carboxylesterase producing bacteria as shown in Fig. 1. The primers used to amplify a ~500 bp portion of the carboxylesterase gene from the IS2 genome. In this experiment CE-f (5′-GAC GCC TGT GTG ATC TGG TTG-3′) and CE-r (5′-CCC AGG TGT TGA GGT ACT CGA-3′) were used as upstream and downstream primers, respectively. Amplification was carried out in 50 µl reactions containing 10 µl of *Taq* polymerase buffer (5×), 1 µl of dNTP (10 µM), 2 µl $MgCl_2$ buffer (25 mM), 0.5 µM of each primer (10 µM), 0.125 µl of DNA *Taq* polymerase (5 *µ*/µM) and 1–5 µl of DNA template. The following program was used: denaturation for 4 min at 95°C; then 30 cycles consisting of 94°C for 1 min for denaturation, 62°C for 1 min for annealing, 72°C for 1 min for extension and a final extension step at 72°C for 7 min. PCR products were resolved by electrophoresis on 1% agarose gels and compared to 1 kb DNA Ladder. Purified PCR products were sequenced by using the same primers and the obtained sequences were compared to other known sequences found in the database using the Blast program (http://www.ncbi.nlm.nih.gov/BLAST/).

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1-MTNPLILEPQKTADACVIWLHGLGADRYDFLPVAEFMQERLLSTRFIMPQ 50
2-MTNPLILEPQKTADACVIWLHGLGADRYDFLPVAEFMQERLLSTRFIMPQ 50
3 MTNPLILEPQKTADACVIWLHGLGADRYDFLPVAEFMQERLLSTRFIMPQ 50
4-MTHPLILEPQKTADACVIWLHGLGADRYDFLPVAEFMQERLLSTRFVMPQ 50
  **:******************************************:***

1-APTRPVTINGGYAMPSWYDIKAMTPARAIDEAQLEESAEQVVALIKAEQA 100
2-APTRPVTINGGYAMPSWYDIKAMTPARAIDEAQLEESAEQVVALIKAEQA 100
3-APTRPVTINGGYAMPSWYDIKAMTPARAIDEAQLEESAEQVVALIKAEQA 100
4-APTRPVTINGGYAMPSWYDIKAMTPARAIDEAQLEESADQIIALIEAQRA 100
  ************************************:*::***:*::*

1-KGINLSRIFLAGFSQGGAVVLHTAYIKWQEALGGVIALSTYAPTFNDQHQ 150
2-KGINLSRIFLAGFSQGGAVVLHTAYIKWQEALGGVIALSTYAPTFNDQHQ 150
3-KGIDLTRIFLAGFSQGGAVVLHTAYIKWQEALGGVIALSTYAPTFNDQHQ 150
4-QGIDLTRIILAGFSQGGAVVLHTAYIKWQEALGGVIALSTYAPTFNDNHQ 150
  :**:*:**:************************************:**

1-LSACQQRTPALCLHGVHDPVVIPSMGRTAFEYLNTWGVAARWHEYPMEHE 200
2-LSACQQRTPALCLHGVHDPVVIPSMGRTAFEYLNTWGVAARWYEYPMEHE 200
3-LSACQQRTPALCLHGVHDSVVIPSMGRTAFEYLNTWGVAARWYEYPMEHE 200
4-LSACQQRTPALCLHGVHDPVVIPAMGRTAFEYLNTWGVAARWQEYPMEHE 200
  ******************.****:**********************  *******

1-VVVEELNDIHEWLAKQLQ 218
2-VVVEELNDIHDWLSKQLQ 218
3-VVVEELNDIHDWLSKQLQ 218
4-VVVAELSDIHDWLSKQLQ 218
  *** **.***:**:****
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Fig. 1 Multiple alignment of amino acid sequences of carboxylesterase genes of different *Pseudomonas putida* strains; 1, *Pseudomonas putida* GB; 2, *Pseudomonas putida* F1; 3, *Pseudomonas putida* KT244 0; 4, *Pseudomonas putida* W619. The forward and the reverse primers were designed based on the highlighted amino acids consensus sequence

Results and discussion

Isolation, characterization and identification of malathion-degrading bacterial strains

Five malathion-degrading bacterial strains (IS1, IS2, IS3, IS4 and IS5) were successfully isolated by using an enrichment technique. A clear halo appeared around colonies capable of degrading malathion, which indicated malathion degradation by these bacteria (Fig. 2).

The morphological and biochemical characterizations of all isolates (Table 1) led to the following identification: isolate IS1 was provisionally identified as a *Pseudomonas* sp., IS2 as *Pseudomonas putida*, IS3 as *Micrococcus lylae*, IS4 as *Pseudomonas aureofaciens*, and IS5 was identified as *Acetobacter liquefaciens*.

PCR amplification and sequence analysis of 16S rRNA gene of IS1 and IS2 isolates

DNA sequence analysis of the PCR products from the 16S rRNA genes of the IS1 and IS2 isolates was carried out. The analysis showed that the 1265 bp of 16S rRNA gene of IS1 isolate was 99% similar to that of alphaproteobacterium BAL284 (GenBank accession no AY972871.1). Also, it scored 99% similarity to that of *Pseudomonas diminuta* (GenBank accession no M59064.1) and *Brevundimonas diminuta*

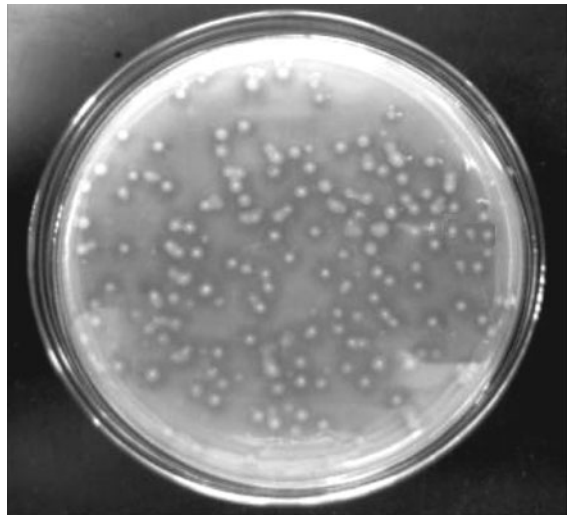

Fig. 2 IS2 bacterial isolate on malathion plate with a clear halo around colonial growth

Table 1 Morphological and biochemical characteristics of isolated bacterial strains

Characteristics	IS1	IS2	IS3	IS4	IS5
Gram staining	−	−	+	−	−
Shape under microscope	Short rods	Short rods	Cocci	Short rods	Rods
Biochemical tests					
Oxidase test	+	+	+	+	+
Gelatin liquefaction	+	−	+	+	+
Starch hydrolysis	−	−	−	−	−
Denitrification	+	−	−	−	−
Arginine dihydrolase	+	+	−	+	+
Utilization of					
Acetate, succinate, lactate, citrate, glycerol, tyrosine, alanine	+	+	−	+	d
Maltose, lactose, starch, isopropanol	−	−	−	+	+
Mannose	−	d	−	+	+
Galactose	−	−	−	d	−
Fructose	+	+	+	−	−
Growth at 41°C	+	−	−	−	−
Growth at 37°C	+	+	+	+	+

+ Positive reaction; − negative reaction; *d* 11–89% positive

strain: c79 (GenBank accession no AB167229.1). The IS1 is considered to be a novel strain belonging to the alphaproteobacteria and it has been named as alphaproteobacterium IS82. The DNA sequence for the 16 s RNA of this strain has been deposited in the GenBank database with accession no. FJ596989.

With respect to the IS2 isolate, it was found that 1391 bp of its amplified 16sRNA gene was 100% identical to that of *Pseudomonas putida*, strain 32zhy (GenBank accession no AM411059.1). Therefore, this strain was named as *Pseudomonas putida* IS168 and its 16sRNA DNA sequence was deposited in the GenBank database with accession no. FJ596988. The alphaproteobacterium IS82 and the *Pseudomonas putida* IS168 (IS1 and IS2) isolates were selected for further identification due to their relatively higher observed growth on malathion as a sole carbon source.

Degradation of malathion in liquid cultures by IS2 strain

Figure 3 shows the GC analysis of the degradation of the malathion. As the Fig. 3 indicates that there was a considerable removal of malathion (50 mg/l) in *Pseudomonas putida* IS168-inoculated media, either in the presence or absence of glucose when compared to

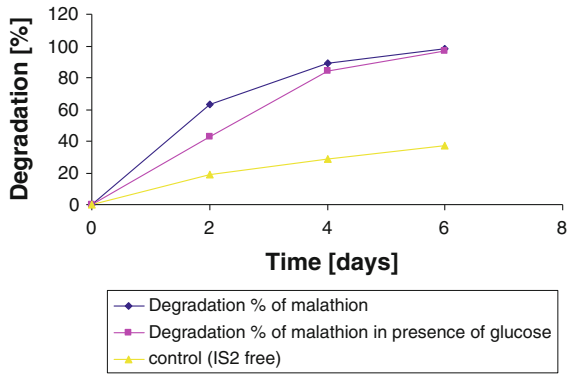

Fig. 3 Degradation and % recovery of malathion after GC analysis

Pseudomonas putida IS168-free media. The malathion degradation in presence of glucose was relatively slow −43% of the rate observed when malathion was present as the sole carbon source. This can be explained by the preferential use of glucose by the isolate as a carbon source instead of malathion during the first 2 days. These results are in consistent with previous reports that degradation of metmitron by *Rhodococcus* sp. was significantly inhibited in MSM supplemented with succinate as the alternative carbon source (Parekh et al. 1994). After four days, the degradation of malathion, either in presence or

absence of glucose, showed no significant difference. Thus, it may be that, malathion consumption by *Pseudomonas putida* IS168 commences after 48 h, when glucose levels have been depleted. Malathion was completely degraded in the inoculated cultures after 6 days. During malathion degradation an increase in optical density occurred, suggesting bacterial growth of *P. putida* (Fig. 4a, b).

Identification of metabolites produced from malathion biodegradation

The spectrum pattern of separated products (Fig. 5) shows molecular ion peaks at *m/z* $[M–H]^-$ 301 and [M–H] 273, which is consistent with the molecular formula of malathion monocarboxylic acid (MMCα or MMCβ), $C_8H_{15}O_6PS_2$ and malathion dicarboxylic

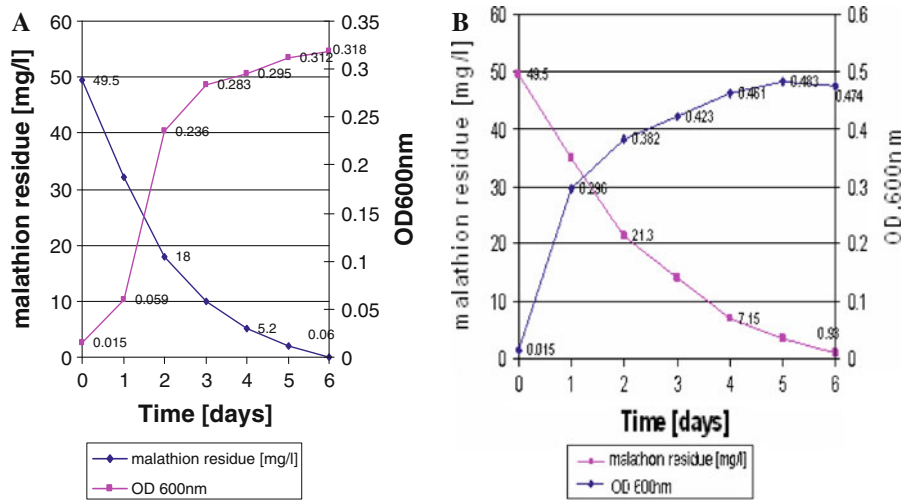

Fig. 4 **a**, **b** Optical density curve of *Pseudomonas putida* (IS2) on malathion (50 mg/l) as a sole carbon source only or in the presence of glucose (0.05%) respectively and concentration of malathion residues

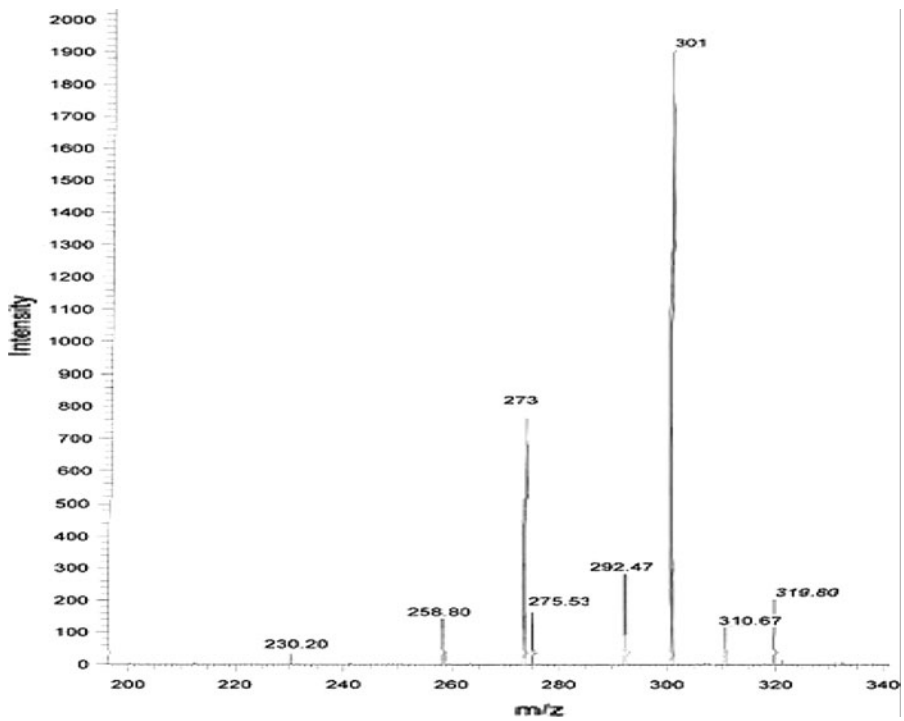

Fig. 5 Mass spectrum chart of malathion metabolites; MMC with *m/z* $[M–H]^-$ 301, MDC with *m/z* $[M–H]^-$273

acid (MDC), $C_6H_{11}O_6PS$. These results are consistent with previous reports which suggest the formation of these metabolites mainly via carboxylesterase activity (Paris et al. 1975; Lewis et al. 1975; Walker 1976; Singh and Seth 1989; Kim et al. 2005).

Utilization of other organophosphorus pesticides by *Pseudomonas putida* 168 (substrate range)

It was reported that certain microorganisms can degrade several xenobiotic compounds (Singh and Walker 2006). The metabolic specificity of OP degrading microorganisms, however, is determined by the chemical resemblance among the OP compounds (Racke and Coats 1987). Our work showed that the intact cells of *Pseudomonas putida* IS168 were able to grow and utilize fenitrothion, diazinon and profenofos when present as a sole carbon sources (50 mg/l) with different optical densities which might indicate a degradation process of these compounds (Fig. 6). However, the strain failed to grow on the pesticide chlorpyrifos. This finding is consistent with the fact that chlorpyrifos is resistant to the phenomenon of enhanced degradation (Racke et al. 1990). It has been reported however, the isolation of chlorpyrifos-degrading bacteria (Li et al. 2007). It was suggested that the accumulation of trichloropyridinol, TCP, (one of the main chlorpyrifos metabolites), which has antimicrobial properties (Racke et al. 1990, Feng et al. 1997 and Cáceres et al. 2007), prevents the proliferation of chlorpyrifos-degrading microorganisms.

All the insecticides tested in this work have phosphotriester bond in their molecular structures, suggesting that hydrolysis at this bond takes place. Due to its broad specificity against a range of OPs, the strain *Pseudomonas putida* IS168 therefore possesses a significant potential to provide a versatile gene and enzyme system that can be used for the remediation of toxic OP products. The other four strains isolated in this study are being investigated in the same manner as *Pseudomonas putida* IS168. We will try to study whether there is a link between the nature of the soil where the strain was isolated and its pesticide degrading ability.

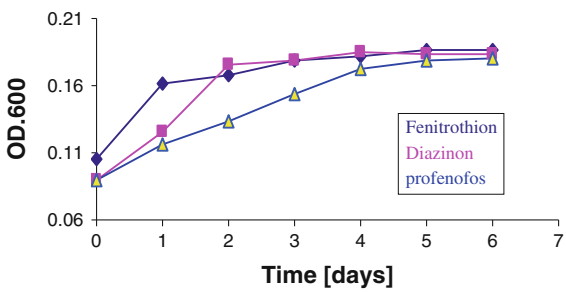

Fig. 6 Optical density curve of IS2 strain on three different OP compounds used as sole carbon sources

Determination of carboxylesterase activity

Malathion biodegradation to malathion mono-acid and malathion diacid may occur through the action of carboxylesterase. Subsequently, this assay was carried out to determine the degree of carboxylesterase

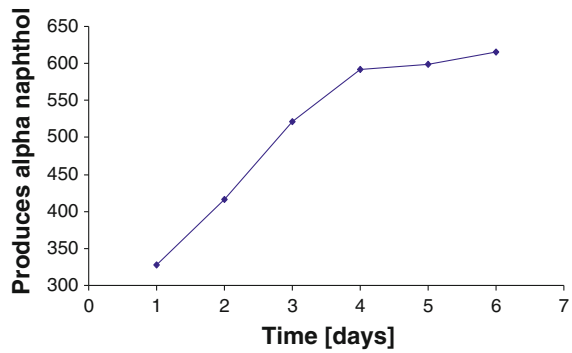

Fig. 7 Esterase activities of culture filtrate of *Pseudomonas putida* (IS2) with malathion as sole carbon source and calculated concentration of produced α-naphthol

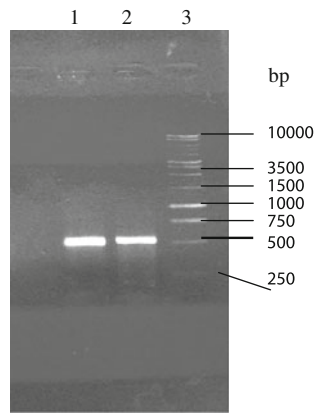

Fig. 8 Agarose gel electrophoresis of the partially amplified CE gene of *Pseudomonas putida* IS2. *Lanes 1, 2* are 10 and 5 µl of the PCR product respectively; *lane 3* is the DNA molecular weight markers as shown in the figure

activity in *Pseudomonas putida* IS168. The results revealed that, when malathion was used as a sole carbon source (50 mg/l), the malathion degradation increased with incubation time, as indicated from the increase in the product of the malathion degradation (α-naphthol) (Fig. 7). It appears from the results that the carboxylesterase activity is related to the malathion degradation. However we can not exclude the involvement of other enzyme(s) in the degradation. The activity of this enzyme showed a non-significant increase after 3 days, which paralleled the growth curve of *Pseudomonas putida* IS168 and the degradation rate of malathion.

PCR amplification and partial sequence analysis of carboxylesterase gene

Based on the fact that the isolated strain *Pseudomonas putida* IS168 has a high carboxylestrase activity we decided to clone (or partially clone) the new carboxylestrase encoding gene. We designed PCR primers based on consensus amino acid homology

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P V A E X L Q E V L L S T R F V M P Q A P T Q P V T I N N G Y A M P S
W Y D I K A L T P G A R A I D E A Q L D A S A Q A V I D L I K Q E Q A
K G V A L S R I I L A G F S Q G G A V V L H T A Y V K W Q E T L G G V
M A L S T Y A P T F T D S T V L S A S Q Q R I R R C
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Fig. 9 Deduced amino acid sequence of the new carboxylesterase, ICE1, from *Pseudomonas putida* IS168

Fig. 10 Multiple alignment of deduced amino acid sequence of carboxylesterase (ICE1) from *Pseudomonas. putida* IS168 and top five closest relative strains: **BOKGP4**, carboxylesterase (EC 3.1.1.1) from *Pseudomonas putida* strain GB-1; **A5W8T9**, carboxylesterase (EC 3.1.1.1) from *Pseudomonas putida* 218 AA strain F1/ATCC 700007; **Q88NB6**, carboxylesterase from *Pseudomonas putida*, strain KT2440; **B1J159**, carboxylesterase (EC 3.1.1.1) from *Pseudomonas putida* strain W619; **Q1I578**, carboxylesterase (EC 3.1.1.1) from *Pseudomonas entomophila* strain L48, **ICE I**; carboxylesterase from our new isolated strain

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B0KGP4    MTNPLILEPQKTADACVIWLHGLGADRYDFLPVAEFMQERLLSTRFIMPQ 50
A5W8T9    MTNPLILEPQKTADACVIWLHGLGADRYDFLPVAEFMQERLLSTRFIMPQ 50
Q88NB6    MTNPLILEPQKTADACVIWLHGLGADRYDFLPVAEFMQERLLSTRFIMPQ 50
B1J159    MTHPLILEPQKTADACVIWLHGLGADRYDFLPVAEFMQERLLSTRFVMPQ 50
Q1I578    MTHPLILEPQKTADACVIWLHGLGADRYDFLPVAEFLQERLLSTRFVMPQ 50
ICE1      --------------------------------PVAEXLQEVLLSTRFVMPQ 19
                                             **** :** ******:***

B0KGP4    APTRPVTINGGYAMPSWYDIKAMTP-ARAIDEAQLEESAEQVVALIKAEQ 99
A5W8T9    APTRPVTINGGYAMPSWYDIKAMTP-ARAIDEAQLEESAEQVVALIKAEQ 99
Q88NB6    APTRPVTINGGYAMPSWYDIKAMTP-ARAIDEAQLEESAEQVVALIKAEQ 99
B1J159    APTRPVTINGGYAMPSWYDIKAMTP-ARAIDEAQLEESADQIIALIEAQR 99
Q1I578    APTRPVTINGGYEMPSWYDIKAMTP-ARAIDEAQLDESAEQVIALVEAER 99
ICE1      APTQPVTINNGYAMPSWYDIKALTPGARAIDEAQLDASAQAVIDLIKQEQ 69
          ***:*****.** *********:** *********: **: :: *:: ::

B0KGP4    AKGINLSRIFLAGFSQGGAVVLHTAYIKWQEALGGVIALSTYAPTFNDQH 149
A5W8T9    AKGINLSRIFLAGFSQGGAVVLHTAYIKWQEALGGVIALSTYAPTFNDQH 149
Q88NB6    AKGIDLTRIFLAGFSQGGAVVLHTAYIKWQEALGGVIALSTYAPTFNDQH 149
B1J159    AQGIDLTRIILAGFSQGGAVVLHTAYIKWQEALGGVIALSTYAPTFNDNH 149
Q1I578    AKGIDLSRIVLAGFSQGGAVVLHTAYIKWQEALGGVIALSTYAPTFTDGL 149
ICE1      AKGVALSRIILAGFSQGGAVVLHTAYVKWQETLGGVMALSTYAPTFTDST 119
          *:*: *:**.****************:****:****:*********.*

B0KGP4    QLSACQQRTPALCLHGVHDPVVIPSMGRTAFEYLNTWGVAARWHEYPMEH 199
A5W8T9    QLSACQQRTPALCLHGVHDPVVIPSMGRTAFEYLNTWGVAARWYEYPMEH 199
Q88NB6    QLSACQQRTPALCLHGVHDSVVIPSMGRTAFEYLNTWGVAARWYEYPMEH 199
B1J159    QLSACQQRTPALCLHGVHDPVVIPAMGRTAFEYLNTWGVAARWQEYPMEH 199
Q1I578    QLSACQQRTPALCLHGVHDPVVIPSMGRTAFEHLNTWGVAARWYEYPMEH 199
ICE1      VLSASQQRIRRC---------------------------------------- 131
           ***.***

B0KGP4    EVVVEELNDIHEWLAKQLQ 218
A5W8T9    EVVVEELNDIHDWLSKQLQ 218
Q88NB6    EVVVEELNDIHDWLSKQLQ 218
B1J159    EVVVAELSDIHDWLSKQLQ 218
Q1I578    EVVVEELNDIHDWLSRQLQ 218
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between different carboxylestrase genes (Fig. 1) as described in Experimental section. The primers CE-f and CE-r successfully amplified a portion of a carboxylesterase gene from *Pseudomonas putida* IS168, producing an amplicon of ~500 bp (Fig. 8).

The obtained PCR product was purified, sequenced and the translated amino acid sequence (Fig. 9) subjected to a search against the NCBI protein database. It was found that the sequence had the highest degree of identity (80%) to a carboxylesterase (EC 3. 1. 1. 1) of *Pseudomonas entomophila* strain L48. Also, it scored significant similarity (79%) to the carboxylesterase of different *Pseudomonas putida* strains such as GB-1, F1/ATCC, KT2440 and W619 strains (Fig. 10).

From all previous data, this isolated PCR fragment has no identical sequence in the protein database. We can report in this work, therefore, the partial cloning of a new carboxylesterase gene from the malathion-degrading bacterium *Pseudomonas putida* IS168 which was isolated from Egyptian soils, to which the name ICE1 is given. Figures 7 and 8 show the predicted partial amino acid sequence of the new carboxylesterase and a multiple alignment of ICE1 to related proteins.

Conclusion

We isolated five strains capable of degrading malathion. We focused our immediate investigation on one of these strains, *Pseudomonas putida* IS168, due to its relatively high activity. The *Pseudomonas putida* IS168 strain was isolated from soils in Egypt that may be very well adapted to the climatic and environmental conditions of the country. The results of the present study therefore, indicate that *Pseudomonas putida* IS168 is a promising candidate for application in the removal of malathion and other OPs in contaminated soils, water and crops in Egypt.

We successfully isolated a fragment containing part of a carboxylesterase gene from the new strain, which had showed 80% sequence homology with other known carboxylesterases. Although it remains to be demonstrated that this enzyme is responsible for the observed degradation of malathion, its activity, together with the results of previous studies, suggest that it merits further study. Therefore, further research is being carried out in our laboratory to clone the complete carboxylesterase gene and to overexpress it in an environmentally friendly host to study the effectiveness of the recombinant bacteria in the removal of OPs pesticides from environments.

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