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Vol. 15(31), pp. 1661-1672, 3 August, 2016
DOI: 10.5897/AJB2016.15273
Article Number: 77CEBAE59837
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Isolation and characterization of two malathion-degrading *Pseudomonas* sp. in Egypt

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Received 13 February, 2016, Accepted 11 July, 2016.

Pseudomonas aeruginosa **and** ***Pseudomonas mendocina*** **degrading malathion were studied. Morphological, biochemical and 16S rRNA genes for bacterial identification were selected. Biodegradation of some organophosphorus compounds with the 2 bacterial isolates was determined by high performance liquid chromatography (HPLC).** ***P. aeruginosa*** **strain completely removed diazinon, malathion and fenitrothion, but not chlorpyrifos within 14 days.** ***P. mendocina*** **strain was not able to degrade malathion, diazinon and chlorpyrifos completely and no significant degradation for chlorpyrifos. The bacterial growth curve showed a steady increase in the two bacterial isolates masses during malathion degradation. The highest growth rates were with yeast extract, glucose and citrate for the 2 isolates, but not with phenol. Shaked high inoculum density with incubation at 30°C of malathion bacterial cultures were found to be the optimum conditions for malathion degradation. Bacterial culture extracts subjected to liquid chromatography/mass spectrometry (LC/MS) analysis revealed that the separated products were malathion monocarboxylic acid and malathion dicarboxylic acid. Molecular characterization of carboxylesterase enzyme revealed that carboxylesterase amino acid sequences of the 2 isolates showed high identity to other carboxylesterase enzymes of** ***P. aeruginosa*** **and** ***P. mendocina,*** **respectively. Phylogenetic analysis showed that** ***P. aeruginosa*** **was localized in a separate branch from other carboxylesterase producing** ***Pseudomonas*** **sp. So, it is suggested that this enzyme is a novel esterase enzyme. Use of pesticide-degrading microbial systems for removal of organophosphorus compounds from the contaminated sites requires an understanding of ecological requirements of degrading strains. The results provided an important insight into determining the bioremediation potential of both strains. But the mentioned bacteria cannot be the aim of bioremediation due to risk of public health hazard, hence these bacteria cannot be used in bioremediation but their purified enzymes could**.

Key words: Biodegradation, carboxylestrase, organopgosphorus pesticides, *Pseudomonas aeruginosa*, *Pseudomonas mendocina*.

INTRODUCTION

Organophosphorus compounds (OPCs) have been used worldwide as plasticizers, petroleum additives and as pesticides since the Second World War. Although, OPCs are biodegradable, they are highly toxic to mammals and other non-target animals (Ragnarsdottir, 2000). Organophosphorus compounds inhibit the acetylcholinestrase resulting in the over-stimulation of acetylcholine receptors in the synapses (Karalliedde and

Senanayake, 1988). Overstimulation of acetylcholine receptors at the cholinergic synapses of autonomic, central nervous systems and neuromuscular junctions causes agitation, hypersalivation, confusion, convulsion, respiratory failure and finally, death (Eddleston et al., 2008).

Biodegradation is one of the natural processes that help to remove chemicals from the environment by microorganisms (Singh et al., 2004; Zhao et al., 2009). Most microorganisms, especially bacteria, have detoxifying abilities (transformation, mineralization and/or immobilization of the pollutants) and also play a crucial role in biogeochemical cycles for sustainable development of the biosphere. Organophosphorous compounds are esters; the principal reactions that occur are hydrolysis, oxidation, alkylation and dealkylation. Hydrolysis of P-O-alkyl and P-O-aryl bonds is considered the significant step in microbial detoxification. Hydrolysis of OPCs greatly reduces their mammalian toxicity (Kaneva et al., 1998).

Malathion is a wide spectrum OPC used in public health and agricultural setting. Due to extensive use of malathion, exposure risk of living organisms including human beings is very high. Malathion degradation by microorganisms has been reported by several items (Goda et al., 2010; Singh et al., 2012; Janeczko et al., 2014).

Co-metabolism is another special phenomenon; it occurs widely in microbial metabolism. Here, the microorganisms transform the desired toxicant compound even though the compound itself cannot work as the primary source of energy for those organisms. To degrade the xenobiotic, the microbes need the presence of primary substrates that can support their growth. The enzymes or coenzymes made to mineralize the primary substrate may possess some activity for other substrate that is significantly known as co-substrate (Karpouzas and Walker, 2000).

There are different types of enzymes responsible for the degradation of OPCs, the most important of them are phosphotriesterases and carboxylesterases (Goda et al., 2010). Carboxylesterases are enzymes found in the α/β-hydrolase fold family that catalyze the hydrolysis of carboxyl esters via the addition of water (Hosokawa et al., 2007). Several researchers isolated and fully characterized carboxylesterase enzymes recovered from different microorganisms (Hosokawa et al. 2007; Li et al., 2007; Goda et al., 2010). Li and colleagues (2007) were able to fuse carboxylesterase enzyme with a green fluorescent protein that made their organism easily detectable in the environment following the degradation of pesticide residues. The main objective of the present study was isolation and molecular identification of bacterial strains capable of degrading some organophosphorus pesticides particularly, malathion and factors affecting its biodegradation. Molecular characterization of the degrading enzymes was also aimed at.

MATERIALS AND METHODS

Samples collection

Samples were collected from different environmental sites from May 2011 to May 2012, Beni-Suef City, Egypt. Agricultural soil samples were collected by removing about 5 cm of soil surface and 10 cm depth and nearly 50-100 ml of agriculture wastewater and domestic sewage samples, respectively, were collected and stored at 4°C till use.

Isolation of degrading organisms from different environmental samples

Organophosphorus compounds (OPCs) such as malathion (MAL), chlorpyrifos (CPF), diazinon (DIZ), and fenitrothion (FEN) were dissolved in the least amount of methanol and completed with water to achieve a concentration of 10 mg ml^{-1}. Ten grams of fine granular soil or 10 ml of sewage water were separately added to 20 mg MAL L^{-1} in minimal salt medium (MSM), incubated at 30°C in a rotary shaker at 150 rpm. After three successive transfers, the enrichment culture was serially diluted and spread on MSM plates containing 100 mg MAL L^{-1}. Discrete bacterial colonies based on morphological properties were selected and sub-cultured to obtain pure cultures.

Inoculum preparation for biodegradation study

As described by Anwar et al. (2009), colony forming units per milliliter (c.f.u ml^{-1}) of this suspension was quantified by the dilution plate count technique and required inoculums were prepared by adding appropriate amount of normal saline.

HPLC analysis of organophosphates degradative capacity of the recovered isolates

Degradative capacities of *Pseudomonas aeruginosa* and *Pseudomonas mendocina* were carried out by measuring the residual MAL in MSM medium. One hundred milliliter of MSM supplemented with 100 µg MAL ml^{-1} was inoculated with 1.5×10^8 c.f.u ml^{-1} bacterial suspension and incubated as mentioned previously for 7 days. Centrifugation to remove bacterial pellets and filter sterilization through 0.22 µm to ensure complete removal of bacterial cells was done. Control experiment was carried out in parallel by 100 ml of MSM with 100 µg MAL ml^{-1} without being inoculated with bacteria and incubated under the same conditions. Five milliliters aliquot from each of the previously mentioned media were centrifuged at 7200 g for 10 min, and extracted 2 times with 50 ml of dichloromethane by vigorous shaking. Dehydration with anhydrous Na_2SO_3 and evaporation of dichloromethane extracts with rotary evaporator at 30°C. The dry residues were dissolved

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in 1 ml acetonitrile and stored at 4°C until HPLC analysis. Twenty microliter of extracted sample were injected in HPLC (Agilent Technologies 1260 Infinity, USA), using Eclipse plus C18 (4.6 ×250 mm 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^{-1}.

Identification of the most active degrader organisms

In addition to morphological and biochemical identification, molecular characterization of bacterial isolates was done (Goda et al., 2010). The DNA extraction was done by GenElute DNA Bacteria Genomic DNA Kit (Sigma-Aldrich, USA). The 16S rRNA gene was amplified using the universal primer pair: 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1525r (5'-AAGGAGGTGWTCCARCC-3'), ordered through, and delivered by Invitrogen, USA. Thermal profile of PCR reaction for amplification of 16S rRNA gene for the most active two isolates was done using Dream Taq Green PCR Master Mix (2X) (Thermo Fisher Scientific Inc., USA). PCR products were visualized by placing on UV transilluminator and were photographed directly. Purification of PCR products from gel were done by GeneJET Gel Extraction Kit (Thermo Fisher Scientific Inc., USA) according to manufacturer protocol and purified DNA was stored at -20°C. Sequencing reactions were performed at Clinilab in MJ research PTC-225 Peltier Thermal Cycler using ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit with AmpliTaq DNA polymerase, following the protocols supplied by the manufacturer. Single-pass sequencing was performed on each template using the above mentioned primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in ABI 3730 x1 sequencer (Applied Biosystem, USA). 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 (Ye et al., 2012).

Bacterial growth curve and factors that may affect the malathion degradation by *P. aeruginosa* and *P. mendocina*

If not otherwise mentioned, the following conditions were adjusted for studying the factors affecting MAL degradation paralleled with control samples at each interval. Each treatment was performed in three replicates. Flasks containing 100 ml of MSM amended with 100 mg MAL l^{-1} were inoculated with 1.5 $\times 10^8$ c.f.u ml^{-1} of isolate and incubated at 30°C on a rotary shaker working at 150 rpm. At different time intervals, samples were taken and then analyzed for the residual MAL concentration by HPLC. Control experiments were carried out in parallel by incubating flasks of the same composition in the same conditions but without addition of the bacteria. These factors included addition of three different carbon sources (glucose, sodium citrate and phenol) and yeast extract as a nitrogen source added separately, at a concentration of 1g l^{-1} to the culture media. Parallel to measurement of MAL biodegradation, growth curve of the 2 isolates in the presence of the 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, 30 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} c.f.u ml^{-1}. 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.

Detection of degradation metabolites of malathion by LC/MS

One hundred milliliter of MSM supplemented with 100 mg MAL l^{-1} was inoculated with bacterial cells suspension with a concentration of 1.5 $\times 10^8$ cf.u ml^{-1} and incubated at 30°C in orbital shaker at 150 rpm for 7 days. Fifty milliliters aliquot from medium was centrifuged at 7200 g for 10 min to remove the bacterial pellets, and extracted 2 times with 50 ml of dichloromethane by vigorous shaking. 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 submitted for HPLC/MS analysis. The equipment was 6120 Quadrupole LCMS, Agilent Technologies with 1260 Quat Pump VL that was used for metabolites detection according to the following conditions: reversed phase C18 column (15 cm × 2.1 mm I.D., 4 µm) at flow rate of 1 ml min^{-1} with injection volume of 100 µl and isocratic mobile phase of acetonitrile/water (70/30, v/v). The mass spectrometer was equipped with electrospray ion source operated in positive and negative polarity mode. The electron spray ion mode was operated under the condition of gas temperature of 350°C and drying gas flow of 12.0 l min^{-1}. The nebulizer nitrogen gas was 35 psi with capillary voltage of 3.0 kV. Full scan signal were recorded within the m/z range of 100 to 1000.

Molecular characterization of carboxylesterase enzyme responsible for organophosphorus degradation

The sequences of the different carboxylesterase genes for *P. aeruginosa* and *P. mendocina* were taken from the NCBI using the online BLASTN program. These sequences were aligned using the multiple sequence alignment tool (ClustalW2) available online (Larkin et al., 2007). Finally, the conserved regions of the genes were determined and then used for primer design. The oligonucleotide primers used in all sets of experiments for carboxylesterase enzyme screening were designed with the online program primer 3 (Ye et al., 2012), corrected manually according to degeneracy of genetic code and ordered from Invitrogen, USA. Primer pairs used in the present study for detection and sequencing of carboxylesterase enzyme for the isolated were: *P. aeruginosa*; Forward: GTAGTCGTGCCAGCCCAC and reverse: CGAACCCCTGATCCTCGATG with product size 574 bp and annealing temperature 56°C. For *P. mendocina,* primer pairs were forward: GTG ATG ACG CAG ATG ATC GT and reverse: CCA TCG TTC CAG CAG TCT CA at annealing temperature of 51°C with product size of 766 bp. Primers preparation, PCR reaction conditions, purification of PCR products and sequencing of amplified PCR products were done as mentioned earlier.

Phylogenetic analysis of 16S rRNA gene and carboxylesterase gene for the degrading isolates

Sequences of 16S rRNA genes of the isolates were aligned with their homologous sequences using ClustalW2 with default settings (Larkin et al., 2007). Phylogeny was analyzed with MEGA version 6.06 software (Tamura et al., 2013) and distances were calculated using Kimura-2 parameter distance model (Kimura, 1980), while clustering was performed with neighbor-joining method (Saitou and Nei, 1984).

For the carboxylesterase gene sequence, ExPASY translate tool was used to convert sequenced nucleotides to their corresponding amino acids sequences (Gasteiger et al., 2003). The translated amino acids sequences were aligned with their homologous sequences using ClustalW2 with default settings (Larkin et al., 2007). Phylogeny was analyzed with MEGA version 6.06 software (Tamura et al., 2013) and distances were calculated using Poisson model (Ranola et al., 2010) clustering was performed with

neighbor-joining method (Saitou and Nei, 1984).

RESULTS

Isolation of active organophosphate-degrading bacteria from environmental samples

A total of 18 bacterial isolates were recovered from thirty-six domestic sewage, agricultural wastewater and soil samples collected from different sites in Beni-Suef City, Egypt. The selected isolates were morphologically and biochemically identified. The most common isolated bacteria were *Pseudomonas* sp. (72.2%), followed by *Enterobacter* sp. (16.6%). On the other hand, only one isolate (5.6%) was isolated from each of the following bacteria: *Serratia* sp. and *Acinetobacter* sp. Regarding the type of the sample, agricultural sewages were the most rich samples with MAL degrading bacteria (49.9%), followed by domestic sewages (38.9%).

Morphological, biochemical and molecular identification of the bacterial isolates

According to the morphological and biochemical results, the selected isolates were preliminary identified as *P. aeruginosa,* isolated from an agricultural sewage sample, and *P. mendocina*, isolated from domestic sewage, and were abbreviated as PA and PM, respectively. To confirm the identified results, their 16S rRNA genes were amplified from their genomic DNA. Sequence blast showed that strain *P. aeruginosa* PA had a high similarity (99%) with *P. aeruginosa* strain ODE5 (Accession number KF420847.1), *P. aeruginosa* strain NRRLB-59992 (Accession number KF18511.1) and *Pseudomonas* sp. strain pDL01 (Accession number AF125317.1). *P. mendocina* PM strain showed a high similarity (99%) with *P. mendocina* strain FB8 (Accession number HQ701687.1) and *P. mendocina* strain PC7 (Accession number DQ178223.1).

Phylogenetic analysis of 16S rRNA sequences of the selected bacterial isolates

Phylogenetic analysis of 16S rRNA gene sequence of *P. aeruginosa* strain showed that our strain PA was closely related to *P. aeruginosa* strain YL 84 that was isolated from compost, a chitinase-producing quorum-sensing bacterium. This study strain was also close to *P. aeruginosa* strain Pa 24 that was isolated from the Arecanut garden soil of Hannoverin coastal Karnataka, India and showed herbicide biodegradation ability. While, there was a great difference with *P. aeruginosa* strain LES like 1 (Liverpool epidemic strain) (Jeukens et al., 2014) (Figure 1a). On the other hand, phylogenetic analysis of 16S rRNA gene sequence of *P. mendocina* strain PM showed that it was the closest to fomesafen-degrading *P. mendocina* strain FB5, which can grow on a medium with 500 mg l^{-1} fomesafen herbicide as a sole carbon source but it was not close to some extent to *P. mendocina* strain PC6, the strain that was able to degrade phenol- and *p*-cresol, as shown in Figure 1b.

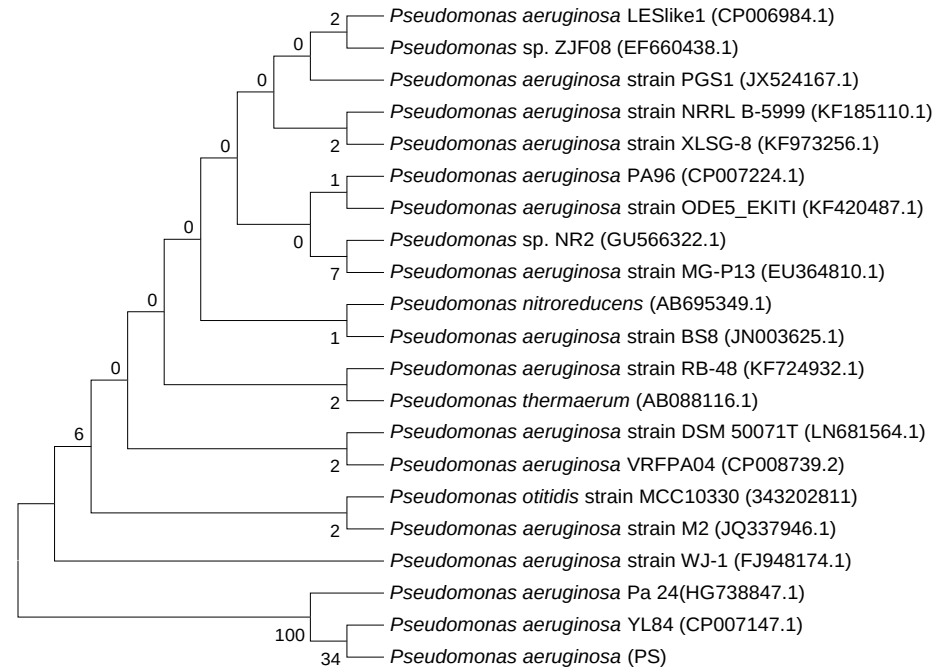
Biodegradation of malathion and other organophosphorus compounds by pure cultures of *Pseudomonas* sp.

Malathion and other three OPCs (DIZ, FEN and CPF) degradation was monitored at different time intervals and their concentrations were determined using HPLC. The obtained results showed that *P. aeruginosa* strain PA grew in the presence of MAL with no lag period and the degradation began within the first 24 h from inoculation and completely disappeared on the 14^{th} d of inoculation. The isolate approximately degraded 91% of DIZ within 7 days from inoculation with almost complete removal in the 11^{th} day and 86 and 96% of FEN were degraded within 11 and 14 days, respectively. Only 56% of CFP was degraded within 14 days (Figure 2a). *P. mendocina* strain PM degradation was less than PA strain where after 24 h from inoculation, only 77.6, 74 and 47% of MAL, DIZ and FEN, respectively, were degraded with no significant degradation for CPF within 14 d of inoculation (Figure 2b).

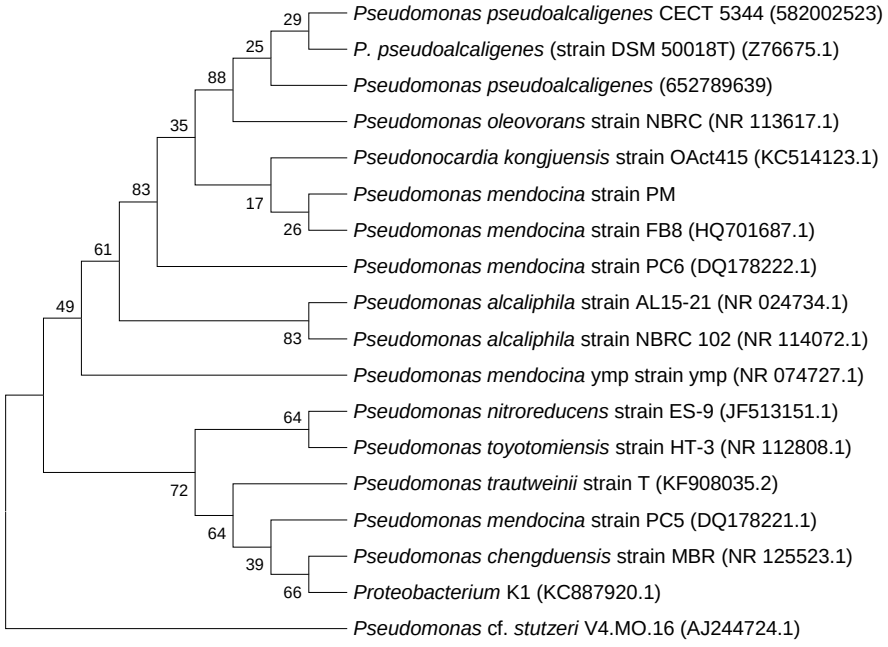
Different factors affecting degradation of MAL by *P. aeruginosa* and *P. mendocina* PM

Factors affecting degradation of MAL by the selected isolates were determined to study the effect of different chemical and physical agents. The bacterial growth curve with OD measurement at 600 nm showed a steady increase in the two bacterial isolates masses. The highest growth rates were with yeast extract, followed by glucose and lastly citrate for the 2 isolates but with phenol and MAL, no significant differences was detected between them (Figure 3).

Simultaneously, the HPLC analysis was carried out to measure MAL concentration with the different carbon sources. Results of Figure 4a and b showed a substantial reduction in the level of MAL concentration in the 2 bacterial cultures. The isolates were capable of degrading MAL in the presence of other carbon sources greater than MAL consumption alone. For *P. aeruginosa* strain PA, it was found that after 4 days, PA strain degraded more than 89, 84 and 81% of MAL in the presence of yeast extract, glucose and citrate, respectively, with almost complete removal within 7 days (Figure 4a). Degradation of MAL by PM strain was also greatly enhanced by the addition of yeast extract, glucose or citrate with significant degradation of up to 90, 83 and 72%, respectively, within 7 days of incubation, with



(a)



(b)

Figure 1. Phylogenetic tree of *Pseudomonas aeruginosa* strain PA (a) and *Pseudomonas mendocina*strain PM (b) 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.

complete removal within 14 days. For phenol, there was no significant difference in the presence or absence of phenol with MAL at any time interval with any bacterial culture (Figure 4b).

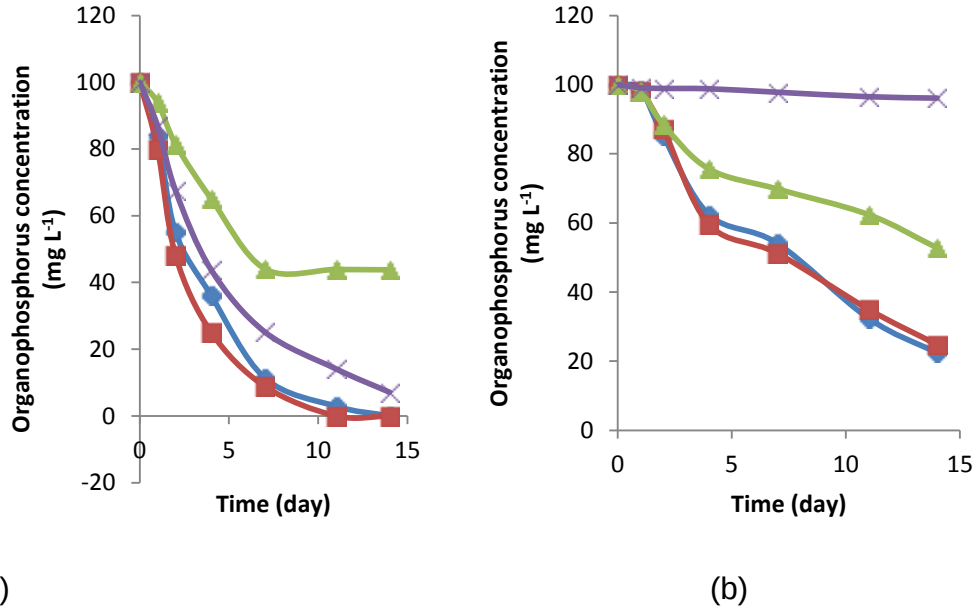


Figure 2. Biodegradation of different organophosphorus compounds by (a)Pseudomonas *aeruginosa* strain and (b)*Pseudomonas mendocina* strain in MSM containing diazinon (■), malathion (♦), fenitrothion (×) and chlorpyrifos (▲) at a concentration of 100 mg.l^{-1} in MSM using each of them as the sole carbon and energy source separately.

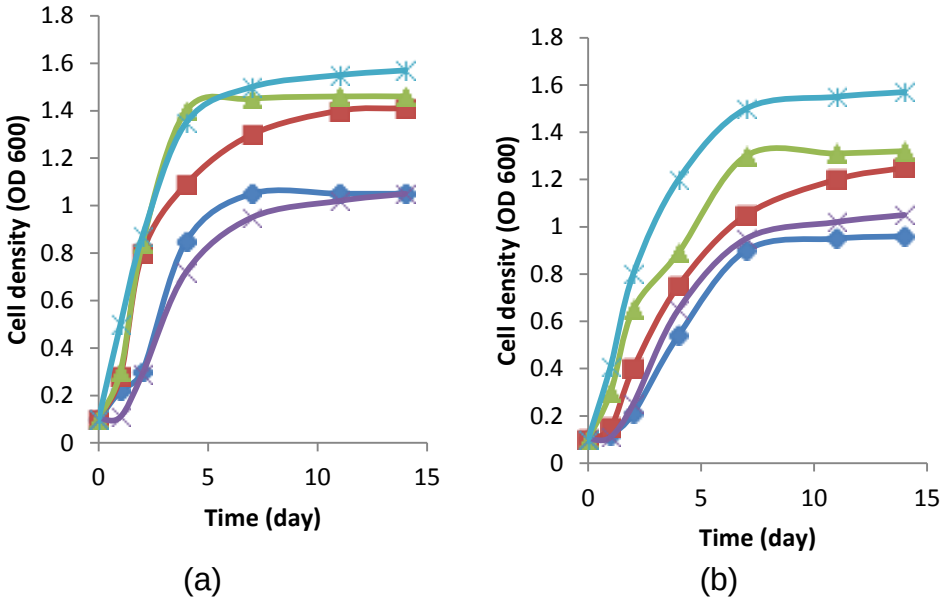


Figure 3. Microbial growth (cell density) of (a) *Pseudomonas aeruginosa* strain PA and (b) *Pseudomonas mendocina* strain PM during malathion biodegradation using it as a sole carbon source (♦) and in presence of different carbon sources as phenol (×), citrate (■), glucose (▲) and yeast extract (✳).

The extent of degradation of MAL increased and lag period decreased with increase in the inoculum density to 1.5×10^{12} c.f.u.ml^{-1} of both PA and PM (Figure 4c and d). *P. aeruginosa* PA strain started MAL degradation immediately after inoculation and at the 4th day, the three bacterial inoculums degraded MAL with the same rate. *P.*

mendocina PM strain at a concentration of 1.5 $\times 10^4$ c.f.u.ml^{-1} could not degrade MAL at all. On the other hand, bacterial concentration with 1.5×10^8 and 1.5×10^{12} c.f.u.ml^{-1} degraded MAL equally with a lag period of 1 day and after 14 days, the concentration reached 30 mg.l^{-1} of MAL (Figure 4d).

At 30°C incubation temperature, PA strain completely degraded MAL within 12 days, while in flasks incubatedat 25 and 37°C, almost 86 and 96%, respectively, of MAL were degraded within 14 d (Figure 4e). Upon incubation of PM strain at 25°C, the lag period was extended to 4 days and only 52% of MAL was degraded within 14 day while at 37°C, the lag period was about 2 days and more than 68% of MAL was degraded. At 30°C, it needed only 24 h to start MAL degradation and approximately 80% were degraded at the end of the incubation period (Figure 4f).

P. aeruginosa strain PA in an unshakable state showed a lag phase extended for 24 h, with a decreased biodegradation ability. Shaking of bacterial cultures with 100 mg MAL l^{-1} resulted in a faster degradation with no shoulder. On the 7th day of incubation, both conditions gave the same rate and extent of degradation and finally disappeared after 14 days of incubation. *P. mendocina* strain PM in resting condition was unable to show biodegradation ability until 4 days of inoculation and the degradation was hardly affected, where only 32% from the initial concentration of MAL was degraded in 14 days. While, in agitation condition, 44% of initial MAL was degraded within 4 days that increased to about 78% after 14 days of inoculation (Figure 4g-h).

Identification of metabolites produced from MAL biodegradation

Bacterial culture extract was subjected to LC/MS analysis to determine the MAL biodegradation metabolites produced from MAL degradation. The spectrum pattern of the separated products showed a molecular ion peak at m/z 330.9 which correspond to MAL. A molecular ion peak at *m/*z 301 and 274 were observed which is consistent with the molecular formula of malathion monocarboxylic acid (MMC) ($C_8H_{15}O_6PS_2$) and malathion dicarboxylic acid (MDC) ($C_6H_{11}O_6PS_2$).

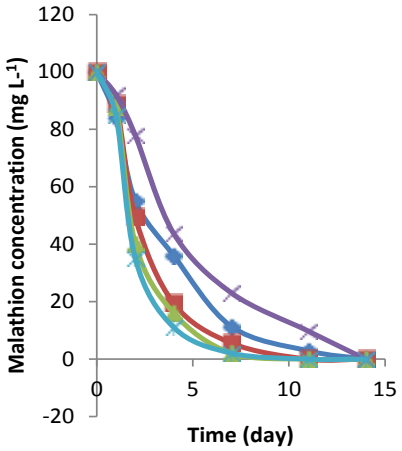
Molecular characterization of carboxylesterase enzyme responsible for organophosphorus biodegradation

Malathion monocarboxylic acid, $C_8H_{15}O_6PS_2$ (MMC) and malathion dicarboxylic acid, $C_6H_{11}O_6PS_2$, (MDC) as biodegradation metabolites produced during degradation of MAL proved the breaking of ester linkage of the MAL by the bacterium. It was suggested that this isolates might contain carboxylesterase enzymes, so specific primers were designed for their detection. The forward and reverse primers successfully amplified genes of ~550 bp for PA and ~750 bp for PM strain. The translated amino acid sequences were aligned using NCBI protein blast tool on (Figure 5). Carboxylesterase amino acid sequence of *P. aeruginosa* PA showed 96% identity to carboxylesterase enzyme of *P. aeruginosa* (WP_003156353.1) and 95% identity to carboxylesterase enzyme of *P. aeruginosa* (WP_034079889.1). Carboxylesterase of *P. mendocina* PM showed 94% identity to carboxylesterase enzyme of *P. mendocina* (WP_013716910.1) and *P. mendocina* (WP_017362547.1) and 90% identity to carboxylesterase enzyme of *P. pseudoalcaligenes* (WP_004424218.1). Phylogenetic analysis with MEGA 6.0 software showed *P. aeruginosa* was localized in a separate branch from other carboxylesterase producing *Pseudomonas* sp. (Figure 6a). Therefore, it is suggested that this enzyme may be a novel esterase, which differed from those previously reported esterase enzymes. As shown in the Figure 6b, carboxylesterase enzyme of *P. mendocina* PM was closely related to carboxylesterase enzyme of *P. mendocina* (WP 013716910), heavy-metal resistant *P. mendocina* strain isolated from France vineyard soil (Chong et al., 2012).

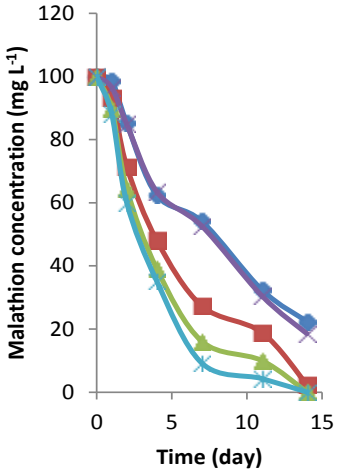
DISCUSSION

Organophosphorus compounds poisoning is a worldwide health problem with about 3 million poisonings and 200,000 deaths annually (Karalliedde and Senanayake, 1988; Sogorb et al., 2004). Bioremediation, which involves the use of microorganisms to detoxify and degrade pollutants, has received increased attention as an effective biotechnological approach to clean up polluted environments. Several chemicals have been successfully removed from soil and aquatic environment using degrading microorganisms such as coumaphos (Mulbry et al., 1996).

In the present study, enrichment culture technique was used to isolate MAL degrading bacteria from polluted samples, where 18 isolates strains were obtained and biochemically identified. Among the isolated *Pseudomonas* sp., *Enterobacter* sp*., Serratia* sp*.* and *Acinetobacter* sp*., P. aeruginosa* and *P. mendocina* were were found to have the highest capacity for degradation of 100 mg MAL l^{-1}. Accordingly, the results in this study suggested that the enrichment culture technique was well suited for the isolation of physiologically and genetically different strains. *Pseudomonas* are a diverse group of bacteria that occur in large numbers in soil, where they are active in mineralization of organic matters. In this study, *P. aeruginosa* strain PA was isolated from agricultural sewage that might be exposed repeatedly to OPCs while, *P. mendocina* strain PM was isolated from domestic sewage that might not be exposed to OPCs.


Malathion concentration (mg L-1)
Time (day)

(a)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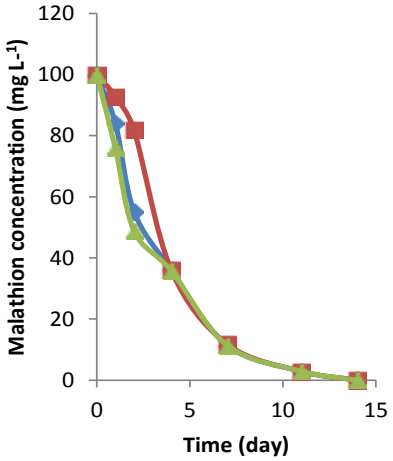
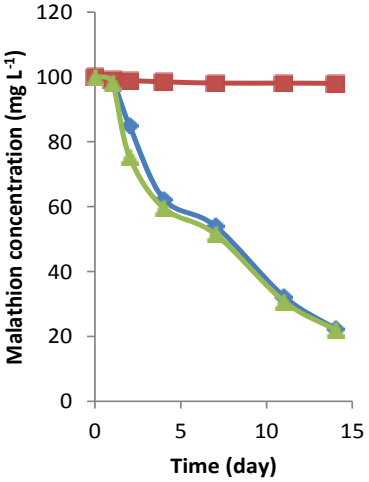
Time (day)

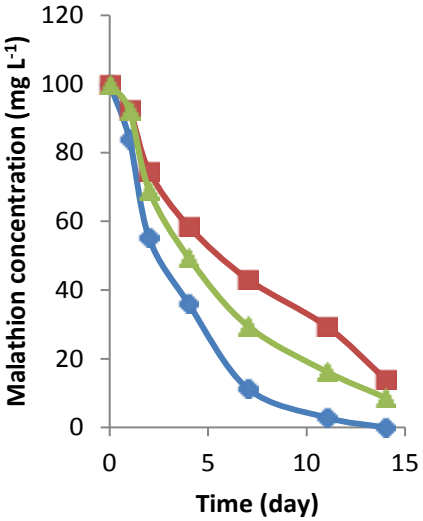
(b)


Malathion concentration (mg L-1)
Time (day)

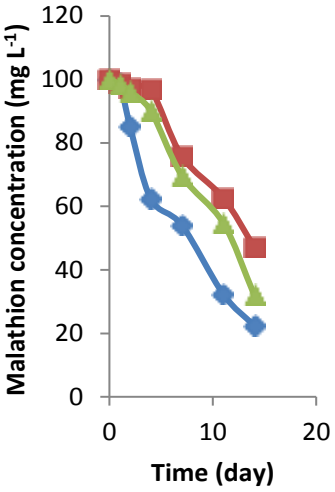
(c)


Malathion concentration (mg L-1)
Time (day)

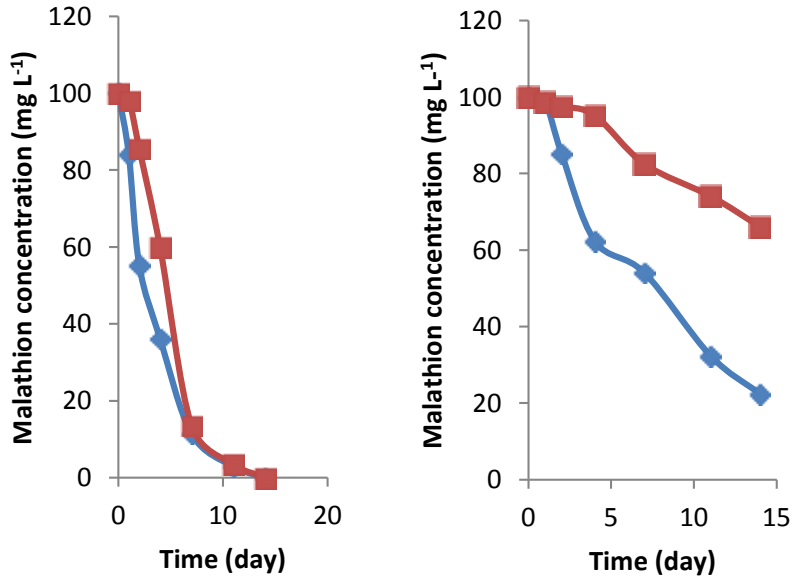
(d)


Malathion concentration (mg L-1)
Time (day)

(e)


Malathion concentration (mg L-1)
Time (day)

(f)



(g) (h)

Figure 4. Effect of different factors on malathion degradation. Effect of different carbon sources on malathion biodegradation by (a) *Pseudomonas aeruginosa* strain PA and (b) *Pseudomonas mendocina* strain PM in MSM medium containing 100 mg. l^{-1} of malathiona sole carbon and energy source (♦), phenol + malathion (×), malathion + citrate (■), malathion + glucose (▲) and malathion + yeast extract (✱). Effect of inoculum density on malathion biodegradation by (c) *Pseudomonas aeruginosa* strain PA and (d) by *Pseudomonas mendocina* strain PM in MSM medium containing 100 $mgl^{-1}$of malathion as a sole carbon and energy source 1.5×10^{4}c.f.u.ml^{-1} (■), 1.5×10^{8}c.f.u.ml^{-1} (♦), and 1.5×10^{12}c.f.u.ml^{-1} (▲). Effect of temperature on malathion biodegradation by (e) *Pseudomonas aeruginosa* strain PA and (f) *Pseudomonas mendocina* strain PM in MSM medium containing 100 mgl^{-1} of malathion as a sole carbon and energy source at 37°C (▲), 25°C (■) and 30°C (♦). Effect of agitation on MAL biodegradation by: (g) *Pseudomonas aeruginosa* strain PA and (h)*Pseudomonas mendocina* strain PM using malathion as a sole carbon and energy source on a rotary shaker at speed 150 rpm (♦) and in stationary case (■).

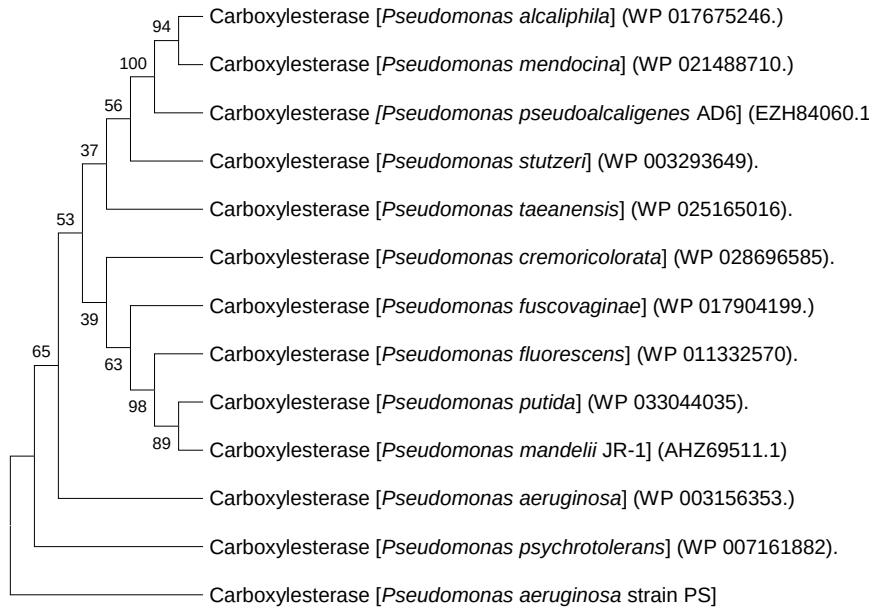
V L V R L Q P V A E A L Q V V L P S T R F I L P Q A P S Q A V T V N G G W V **Met** P S
W Y D I L A F S P A R A I D E D Q L N A S A D Q V I A L L D E Q R A K G I A A E R I I L
A G F S Q G G A V V L H T A F R R Y G K P L G G V L A L S T Y A P T F D D L A

(a)

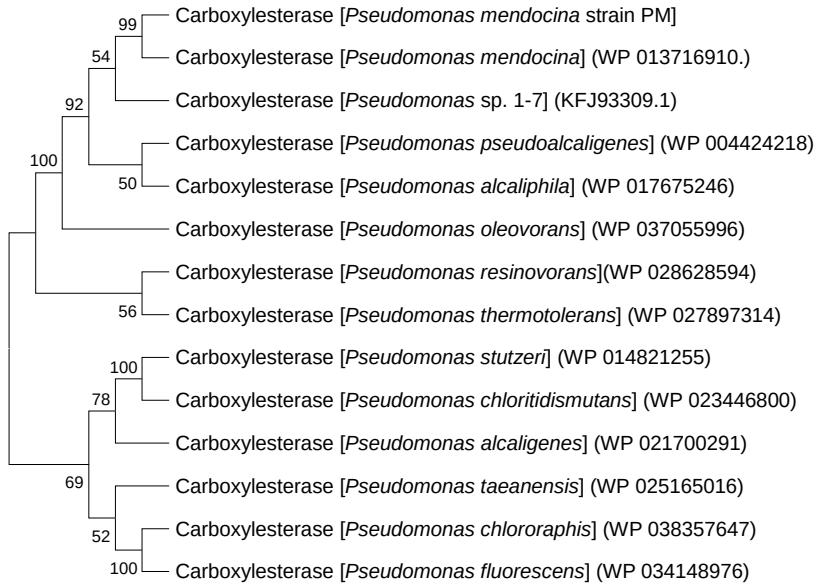
Met T A P **Met** I L Q P P Q T A D A S V I W L H G L G A D R Y D F L P V A E **Met** L Q E R
L P T T R F I L P Q A P T R P V T I N G G W S **Met** P S W Y D I L A **Met** S P A R A I D Q A
Q L D E S A D Q V I A L I E A E R E S A I A A E R I V L A G F S Q G G A V V L H T A F L
R Y P E T L G G V L A L S T Y A P T F S D D **Met** Q L A D T K R Q L P V L C L H G R F D
D V V T P D **Met**G R A A Y D R L H A S G V Q V Q W R D Y P **Met** T H E V L P E E I R D
I A E W L G Q L L A

(b)

Figure 5. Deduced amino acid sequence of carboxylesterase enzymes of (a) *Pseudomonas aeruginosa* PA; (b) *Pseudomonas mendocina* PM, using ExPASY translate tool of sequenced nucleotides.



(a)



(b)

Figure 6. Phylogenetic analysis of carboxylesterase enzyme of (a) *P. aeruginosa* PA (b); *P. mendocina* PM 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.

Prakash and his colleaagues (1996) observed that a pesticide can be degraded rapidly in the soil from a field to which it had never been applied before but which had been exposed to a pesticide from the same chemical group; this phenomenon is known as cross-adaptation.

Phylogenetic analysis of 16S rRNA gene sequence of *P. aeruginosa* strain PA showed that this strain PA was closely related to *P. aeruginosa* strain YL 84 (a chitinase-producing quorum-sensing bacterium) (Chan et al., 2014) and *P. aeruginosa* strain Pa 24 isolated from Areca nut

garden soil and showed herbicide biodegradation ability. The present study results were confirmed by Singh (2009) who reported that the bacteria with the capacity to degrade OPCs were more closely related than those without degrading capability. Phylogenetic analysis of 16S rRNA gene sequence of *P. mendocina* strain PM showed that it was the closest to fomesafen-degrading *P. mendocina* strain FB5. The phylogenetic tree showed sister linage between sequences of xenobiotic degrading microorganisms. The same results were obtained by Cabrera et al. (2010) who reported that fenamiphos degrading bacteria were more closely related than others, and suggested that nematicide degrading bacteria were grouped within a common microbial population which can be of great significance for the soil ecology.

The two bacterial strains isolated in this study were able to utilize MAL as a sole carbon and energy source. In this study of MAL biodegradation, in the presence of yeast extract, glucose or citrate as additional carbon sources, there was a marked increase in the bacterial biomasses for the two bacterial isolates. The findings revealed that *P. aeruginosa* strain PA and *P. mendocina* strain PM preferred to utilize MAL even in the presence of rich nutrient environment and its degrading ability was positively influenced by the presence of supplementary nutrient sources. The preferrential use of MAL in the presence of additional carbon sources was attributed to: 1) MAL degrading enzymes in bacteria were expressed even in the presence of readily available carbon sources; 2) The presence of favorite carbon sources accelerate cell growth, reduce the toxicity of MAL and drastically promoted the degradation of pesticides and 3) when a culture medium contained two compounds, the microorganisms preferrentially utilize the most assimilable and the most concentrated until the concentration approached equality, at which time both sources were utilized simultaneously. In the present case, the first sample was taken after 24 h which was considered as a sufficient time for the complete removal of favourable nutrients, at this time, the equillibrium state was reached with high inoculum density. On the other hand, Goda et al. (2010) found that MAL degradation in the presence of other carbon sources was relatively lower than when MAL was present as a sole carbon source for the first 2 days and after 4 days of the degradation was the same in both cases. They explained the preferrential use of favorable carbon sources instead of MAL. Similar findings with this results had been reported for *P. putida* epII (Karpouzas and Walker, 2000), where the degradation of ethroprophos was inhibited by succinate or glucose. In other studies, the addition of sodium acetate or glucose did not affect the degradation of pentachlorophenol by *P.mendocina* NSYSU (Kao et al., 2005), neither did supplementation with glucose improved degradation of phenanthrene (Zhao et al., 2009). Thus, inclusion of other carbon sources may or may not affect the biodegradation of the insecticides by bacteria. The gradual consumption of MAL was accompanied by a consistent increase in culture density, which reached maximum once MAL had been completely consumed, suggesting that bacterial growth was driven by MAL catabolism. This result was similar to those reported by Zhao et al. (2009).

The obtained results showed that MAL was degraded by *P. aeruginosa* strain PA during incubation with the three intial inoculum densities tested (1.5×10^4, 1.5×10^8 and 1.5×10^{12} c.f.u.ml^{-1}), while MAL degradation by *P.mendocina* strain PM in cultures at a concentration of 1.5×10^4 c.f.u.ml^{-1} could not. Similar results were obtained by Karpouzas and Walker (2000) who found that *Sphingomonas* strain could not degrade cadusafos when the organism was introduced in soil at a density of 4.3×10^4 cells g^{-1}.

Incubation temperature is an important factor affecting the survival of the organism and degradation of the organophosphorus. Incubation temperature of 30°C could be suggested to be the optimum for the growth of the organism and production of degrading enzymes of *P.aeruginosa* and *P. mendocina* giving higher extent of MAL degradation by these isolates. Studies by Hong and colleagues (2007) reported that *Pseudomonas* sp. and *Burkholderia* sp. were able to degrade fenitrothion over a temperature range of 20-40°C with optimum degradation at 30°C. Yang et al. (2008) showed that 30°C is the optimal temperature for heterologous expression of methyl parathion hydrolase enzyme (MPH). They found that 30°C is optimal for the formation of properly folded enzyme and inhibition of ATP-dependent and ATP-independent proteolysis. Also, Kaneva and co-workers (1998) found that cultures expressing organophosphate hydrolase enzyme (OPH) constitutively on the surface did not survive at 37°C and could only be grown at 30°C or lower temperatures.

In the present study, analysis of MAL bacterial culture by HPLC/MS was carried out to identify the metabolite(s) produced due to MAL degradation. It was found that the spectrum pattern of the separated products showed molecular ion peaks, suggesting that the primary degradative pathway of MAL might be due to the activity of esterase enzyme (Goda et al., 2010). From HPLC/MS analysis for identification of the metabolites produced from MAL degradation, it was concluded that carboxy-lesterase enzyme might be the enzyme responsible for the degradation of MAL.

Sequence analysis in the present study of carboxylesterases enzymes of *P. aeruginosa* strain showed a high similarity with carboxylesterase enzyme of *P. aeruginosa*. However, there was no homology with the previously described carboxylesterase enzyme or with other recognized hydrolases that were isolated from mammals or microorganisms. The finding suggested that carboxylesterase in *P. aeruginosa* strain is a novel esterase enzymes that differed from those previously mentioned.

This study carboxylesterase enzyme maybe coded by

chromosomal genes, suggesting that it should be a stable trait, once more emphasizing the potential for use of these isolates in bioremediation process. The suggestion was almost confirmed by the stability of its degradation power even in the presence of other favorable carbon sources. A recent study by Farrugia et al. (2013) confirmed the result that carboxylesterase enzyme was encoded by chromosomal genes. On the other hand, many of the genes for xenobiotic transformation were carried on mobile elements such as plasmids (Pieper and Reineke, 2000) or megaplasmid of 60 Kb in *Bacillus* sp. (Niazi et al., 2001).

For future work, there is a need for further research on some other parameters that may affect the degradation of organophosphorus compounds such as pH and use of different concentrations of the same organic substrate. As shown, these bacterial enivronmental biohazards to carboxylesterase enzyme purification and stability is one of the aims of future research and attempt of enzyme cloning in a competent *Escherichia coli* will also be the authors' concern.

Conflict of Interests

The authors have not declared any conflict of interests.

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