Biodegradation of Chlorpyrifos and Diazinon Organophosphates by Two Bacteria Isolated from Contaminated Agricultural Soils

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Abstract

Introduction: The organophosphate insecticides such as chlorpyrifos and diazinon have been widely used to control insects in home, agriculture, and veterinary. These compounds are a threat to public health and the environment; on the other hand, they have low degradation rate and therefore can be stable for a long time in soil. A major factor determining the fate of organophosphate insecticides in soil and water is biodegradation.

Materials and methods: Two diazinon and chlorpyrifos degrading bacterial strains were isolated from pesticides contaminated soils. The isolated bacterial strains were identified by 16S ribosomal RNA gene and fatty acid methyl ester analysis.

Results: Strong correlation was seen between microbial growth and the two organophosphates degradation. On average, bacterial strain 1 and 2 degraded 88.27% and 82.45% of initial applied diazinon in medium and degraded 81.07% and 88.35 % of initial applied chlorpyrifos during 20 days, respectively. The isolated bacterial strains were identified as *Acinetobacter* and *Pseudomonas sp*. The highest diazinon degradation were found by *Acinetobacter* and the highest chlorpyrifos degradation were found by *Pseudomonas* when cultivated in the mineral salt medium.

Discussion and conclusion: The identified pure bacterial strains utilized chlorpyrifos and diazinon as a source of carbon. They were able to degrade most of the parental molecule in 20 days. Therefore, the isolated bacterial strains may have the potential for use in the bioremediation of diazinon and chlorpyrifos-contaminated soils.

Key words: Bacterial Degradation, Bioremediation, Chlorpyrifos, Diazinon

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Introduction

It is reported that during 2006–2007, about 5.2 million tons of pesticides were applied to world crops annually for pest control which constituted 17 % insecticides. Generally, 1 % of the total applied pesticides get to the target pest (1). So, a large amount of them remain unused and enter into environment specially soil (2).

Because of high stability and toxicity in our ecosystem, most of them are banned all over the world. However, chlorpyrifos and diazinon are the most commonly used organophosphate pesticides for their broad spectrum insecticide as a part of pest control strategy. They can adversely affect ecosystems by serious environmental pollution and also can cause serious health problems by inhibiting acetyl cholinesterase, an enzyme essential for normal nerve impulse transmission (3, 4).

Depending on temperature, pH, and sunlight as well as the presence of microorganisms, diazinon has a half-life of about 70 h to 12 weeks in water (5). Chlorpyrifos also undergoes little biodegradation and persists in soil at neutral pH. The hydrolysis half-life of chlorpyrifos at 25°C in neutral conditions ranged between 35 and 78 days. At termiticidal rates, chlorpyrifos was persistent in the environment about 175–1,576 days and remained effective up to 17 years (6, 7).

Microbial degradation is the major mechanism of pesticide degradation and determining the fate of diazinon and other organophosphate insecticides in the environment. Many authors indicated that the bacterial strains belonging to the different taxonomic group have a great degradation potential of the organophosphate insecticides and other pesticides (8, 9).

A chlorpyrifos-degrading bacterium *Bacillus pumilus* strain has recently been isolated from soil. It has been proposed for bioremediation purposes (10). It was also reported that *Flavobacterium sp*. (11) and *Escherichia coli* clone (12) co-metabolically degraded chlorpyrifos in liquid media. However, these organisms do not utilize it as an energy source. Yang et al. (2005) isolated *Alkaligenes faecalis* DSP3, which was capable of degrading chlorpyrifos and 3,5,6-trichloro-2-pyridinol (13), and also bacterial strain *Klebsiella sp*. isolated from an activated sludge of wastewater treatment plant was able to degrade chlorpyrifos as a sole carbon source (14). *A Sphingomonas sp.*by hydrolyzing chlorpyrifos to 3, 5, 6-trichloro-2-pyridinol utilized it as a sole source of carbon for growth (15). In addition, Rani et al. (2008) reported that a single soil bacterial isolate designated as MS09 *(Providen ciastuartii)*, was capable of utilizing chlorpyrifos as a single carbon source (16). In this regard, Cycoń et al. (2009) isolated three bacteria using diazinon as a sole carbon source, which, within 14 days, 80–92% of the initial dose of insecticide was degraded by the isolates (3). It has been also reported that *Pseudomonas aeruginosa sp*., *Clavibacterm ichiganense sp*. (2) *Arthrobacter atrocyaneus sp*., *Bacillus megaterium* sp. and *Pseudomonas mendocina* (17) degraded monocrotophos as a sole carbon or phosphorus source in liquid media.

The use of organophosphate insecticides has caused surface and groundwater pollution in some parts of Iran. Therefore, the purpose of this study was to isolate native bacterial strains from polluted agricultural soils in semiarid regions and to characterize their degradation potential. To ascertain this ability, the utilization of diazinon and chlorpyrifos as a sole carbon source in the liquid medium by isolated bacteria was investigated.

Material and Methods

Chemicals and reagents: Technical grade of chlorpyrifos (97 % purity) and diazinon (97 % purity) was procured from Razi Shimi Khoram co (Iran). A Stock solution of 100 mgL^{-1} was prepared in acetone. All the other chemicals employed in the present study were obtained from Merck, Germany. Microbiological media was procured from Himedia. Taq DNA polymerase and DNA ladder were from Thermo Scientific. API20 NE strip was purchased from Biomeriux.

Soil sampling: Two composite soil samples were collected from the contaminated areas of various gardening farms in Damavand in Tehran province and in Hamadan province, Iran, which were sprayed with chlorpyrifos and diazinon to control infestation during the past five years. The samples were collected using spade from the upper layer (0-10 cm) of soils. The collected soil samples were stored in airtight polythene bags and stored at 4 °C until use. Then, each soil sample was gently air-dried to the point of soil moisture content of 20 % (W/W) which was suitable for sieving. Then the soils were sieved through 2 mm mesh before use. Based on texture analysis, the soils were classified according to FAO and USDA System, pH value of the aqueous soil extracts (1:2, w/v) were measured with a glass electrode by a Jenway pH-meter at 20 °C. Soil electrical conductivity (EC) was measured in a 1:5 soil: water extract. Organic carbon (OC) was analyzed by dichromate oxidation and titration with ferrous ammonium sulfate (18). Detailed physical and chemical properties of the soil are presented in Table 1.

Table 1- Some physical and chemical properties of the soil samples

Soil location	Texture	Organic carbon	CCE	EC in 1:5 soil: water extract	pH in 1:2 soil: water
		g/100g	%	(dS m^{-1})	
Hamadan	Loam	0.90	2.70	0.14	7.40
Damavand	Clay loam	0.79	10.87	0.89	7.70

CEC- cation exchange capacity, CCE-calcium carbonate equivalent.

Bacterial isolation and culture conditions: The mineral salt medium (MSM, pH 7.2) was prepared by adding 0.5 g K$_2$HPO$_4$, 0.1 g KH$_2$PO$_4$, 0.2 g MgSO$_4$, 0.001 g FeSO$_4$, 0.01g Ca(NO$_3$)$_2$, 0.008g Co(NO$_3$)$_2$ into 1000 mL of distilled water. After autoclaving (121 °C, 20 min) and cooling, diazinon was added in the form of acetone solution as the sole carbon source to give the final concentration of 50 mgL^{-1}. After 24 h of shaking and solvent evaporation, a stock culture medium was prepared by transferring 10 g of the soil into the flask containing 100 mL MSM and cultivated at 30 ± 1 °C on a rotary shaker (120 rpm) for 96 h and kept from the light to avoid photo-degradation of diazinon. Then 10 ml of medium was removed from the flask and inoculated into fresh MSM with diazinon at 50 mgL^{-1}.

Bacterial strains were isolated from the contaminated soils by plating 10-fold dilutions of the liquid medium onto MSM agar supplemented with diazinon at the concentration of 50 mgL^{-1} as the sole carbon source and incubated at 30 ± 1 °C for 7 d. The colonies on the plate observed in about 3-5 days. Based on morphological properties the individual bacterial colonies were selected and sub-cultured to obtain a pure culture. Repeated sub-culturing was done to get pure cultures. Among the bacteria isolated, two bacteria were selected for further assessment. Strain 1 (BS1) was isolated from sampled soil from gardening farms in Hamadan province, and strain 2 (BS2) was isolated from gardening Damavand farms in Tehran province.

In order to assess the ability of the isolated bacteria to degrade chlorpyrifos,

the cultivated bacteria were transferred into MSM medium with chlorpyrifos, and bacterial growth or cell density (OD$_{600nm}$) and pesticide biodegradation were monitored.

Identification of the degrading bacteria: Biochemical characteristics of the isolates were determined by API20 NE tests. Then, in order to identify the two isolates by phospholipid fatty acid, pure cultures were streaked on Trypticase Soy Broth (TSB) agar and incubated at 28 °C for 24 h. A loopful of cells was harvested and placed into a clean screw-capped glass tube. Fatty acids were saponified by boiling at 100°C with sodium hydroxide/methanol mixture and methylated with a methanol/hydrochloride acid mixture. Methyl esters of the fatty acid were extracted with hexane and methyl tert-butyl ether followed by a base wash with sodium hydroxide. The purified methyl esters were analyzed by gas chromatography. The prepared samples were analyzed using the RTSBA6 library, which aimed for environmental bacteria and contains fatty acid profiles for aerobes grown on TSA agar (19).

In addition, the identification of one of the isolates was done by sequencing of 16s rRNA gene. Fast-growing and more efficient species (BS1) was grown overnight on Luria Broth (LB) medium. The cells were pelleted by centrifugation at 6,000 × g for 5 min, and the DNA was extracted by CTAB-NaCl method. The 16S rRNA genes of the bacterial isolates were amplified with a set of universal primers that allow amplification of most bacterial rRNA genes. The primers 27f (5′ AGAGTTTGATCCTGGCTCAG 3′, forward) and 1492r (5′ TACGYTACCTTGTTACGACTT 3′, reverse) were used (Lane et al., 1985). PCR conditions were set as follows: initial denaturation at 94°C for 5 minutes, 30

cycles consisting of denaturation at 94°C for 45 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1:20 min, and final elongation at 72°C for 7 minutes. After amplification, 5 µl of each reaction mixture was run on 0.1% agarose gel to confirm the size and purity of PCR products. The DNA was then purified with a QIA quick PCR purification kit (Qiagen, United Kingdom) following the protocol provided by the supplier. Purified PCR products were subjected to sequencing. The gene sequences were compared with the most similar sequence available in the Gen bank nucleotide sequence database using the NCBI blast program.

Inoculum preparation: To prepare bacterial inoculum, the pure culture of each isolate, obtained from individual colonies, were inoculated onto plates with MSM agar supplemented with diazinon at the concentration of 50 mgL^{-1} and incubated at 30 ± 1 °C. The isolate was cultured in the 250 mL flask containing 100 mL MSM medium supplemented with 50 mgL^{-1} diazinon. Flasks were shaken at 150 rpm, at 30°C and the cell density OD$_{600nm}$ was measured every hour by using UV–Vis Spectrophotometer (Cecil). At the same time, 50 µl of the supplement was cultivated in Luria Broth (LB) agar for plate count method. The figure of cell numbers against OD$_{600nm}$ was plotted. For the whole experiment, approximately 10^{9} cells per ml were used for inoculation.

Biodegradation of the two organophosphates in liquid medium: Growth experiments with diazinon and chlorpyrifos as a sole carbon source were performed in 500 mL Erlenmeyer flasks containing 200 mL of sterile MSM. Different concentrations of diazinon and chlorpyrifos were introduced in the form of acetone solution to give the concentration of 0, 50, 100, 150 mgL^{-1}. After 24 h of shaking and solvent evaporation, the bacterial suspension of

each isolate prepared (described in the previous section) was inoculated into MSM to give the final concentration of $3*10^6$ cells mL^{-1} (3). Each treatment was tri-replicated, with a non-inoculation treatment as the control. Flasks were incubated at 30 ± 1 °C on a rotary shaker (120 rpm) and kept from the light to avoid photo-degradation of insecticides. Samples of liquid medium were periodically removed aseptically for the growth rate of bacteria as well as for chemical analyses to determine the concentrations of insecticide. The growth of isolates was recorded spectrophotometrically by taking OD at 600 nm by using UV–Vis Spectrophotometer (Cecil). Flasks were incubated in a rotary incubated shaker for 20 days.

Extraction of Pesticide: The organophosphates were extracted using dispersive liquid–liquid micro-extraction (20). According to the method, 5 ml of bacterial suspension was placed in a 10 ml screw cap glass test tube with a conic bottom. 1200 µL of acetone (as disperser solvent) containing 200 µL carbon tetrachloride (CCl_4) (as extraction solvent) was injected into a sample solution by using 2.00 mL syringe and then the mixture was gently shaken. A cloudy solution (water, acetone, and carbon tetrachloride) was formed in the tube. Then, the mixture was centrifuged for 3 min at 6000 rpm. Accordingly, the dispersed fine particles of extraction phase were settled at the bottom of conical test tube. The settled phase was removed using a 50.00 µL microsyringe (zero dead volume, Hamilton) and transferred to a microtube, then the extracts were concentrated to near dryness and suspended in acetone and injected into HPLC. A blank was also maintained to assure the quality control of the experiments.

Quantification of the insecticides by HPLC: The residue of insecticides was analyzed using chromatography (HPLC Cecil 1100) with a Zorbax SB-C18 column (250 × 4.6 mm 2, 5Rm). The column temperature was 25°C. The mobile-phase reservoirs contained a 30/70 v/v% mixture of water and acetonitrile with a flow rate of 2 mL min^{-1}. The wavelength used for detection of chlorpyrifos and diazinon was 290, 254 nm, respectively. The retention time was 11.30 min for diazinon and 18.1 min for chlorpyrifos.

Data analysis: The degradation rate constant (k) was determined using the algorithm $C_t/C_0 = e^{-kt}$, where C_0 is the amount of insecticides in the soil at time zero, C_t is the amount of insecticides in soil at time t, k is the degradation rate constant (day^{-1}), and t is the degradation period in days. The theoretical DT_{50} values were calculated from the linear equation obtained from the regression between Ln (C_t/C_0) of the chemical data and time.

Results were also evaluated by analysis of variance, and statistical analyses were performed on three replicates of data obtained from each treatment. The significance ($p < 0.05$) of differences were treated statistically by repeated measurement test and assessed by the post hoc comparison of means using lowest significant differences (Duncan) test using program Statistica 6.0.

Results

Bacterial growth in mineral salt medium with diazinon and chlorpyrifos as sole carbon source: Table 2 shows the analysis of variance (mean square) and the significant ($p<0.01$) effects of Bacterial Strain (BS), Organophosphate Concentration (OC), Incubation Time (IT) and their interactions on the growth of bacteria (OD_{600nm}) in the MSM medium with diazinon and chlorpyrifos as a carbon source.

Table 2- Analysis of variance (mean square) of the effect of bacterial strain (BS), organophosphate concentration (OC), incubation time (IT) and their interactions on the growth of bacteria (OD_{600nm}) in the MSM with diazinon and chlorpyrifos as a source of carbon

Source of variation	diazinon		Chlorpyrifos	
	Df	Mean square	Df	Mean square
BS	1	2.06**	1	0.445**
OC	3	4.640**	3	5.042**
BS*OC	3	0.415**	3	0.061**
Error	16	0.002	16	0.009
IT	4	1.054**	4	0.640**
BS*IT	4	0.161**	4	0.021**
OC*IT	12	0.160**	12	0.112**
BS*OC*IT	12	0.028**	12	0.015**
Error	64	0.003	64	0.004

** Mean square of the treatment is significant at the 0.01 level

As seen in Figure 1, by increasing the concentration of the two organophosphate insecticides in culture medium from zero to 150 mgL^{-1}, OD_{600nm} also increased. As diazinon concentrations increased up to 150 mgL^{-1}, OD_{600nm} in BS1 increased from 0.02 to 1.43 and in BS2 from 0.03 to 0.93 after 20 days of incubation. In the case of culture containing chlorpyrifos, increasing concentrations up to 150 mgL^{-1}, resulted to an increase in the $OD_{600 nm}$ for BS1 from 0.02 to 1.04 and for BS2 from 0.02 to 1.16 after 20 days of incubation. Therefore, BS1 had the highest $OD_{600 nm}$ in MSM containing 150 mgL^{-1} diazinon and BS2 had the highest $OD_{600 nm}$ in MSM containing 150 mgL^{-1} chlorpyrifos.

According to Figure 1 (a, b), on average, in three concentrations of culture containing diazinon and chlorpyrifos at different incubation times, the OD_{600nm} of BS1 was the highest at 5th day of incubation, and at the first day of incubation, OD_{600nm} was lowest. According to Figure 1 (a, b), on average in three concentrations of culture, the maximum OD_{600nm} of BS2 culture was achieved after 9th day of incubation when

diazinon and chlorpyrifos were used as the sole carbon source in MSM media. In contrast, a non-inoculated control showed no change in OD_{600nm} during 20 days of incubation.

Also, in Figure 1 the growth profiles of two bacteria in MSM media containing diazinon and chlorpyrifos as sole carbon source in three organophosphate concentrations were compared. During the incubation period, BS1 in MSM containing diazinon had better growth compared to other strain. However, BS2 in MSM containing chlorpyrifos had better growth compared to BS1.

The capability of isolated bacteria for biodegradation of the two organophosphates in mineral salt medium:

Degradation of diazinon and chlorpyrifos by the isolated bacteria was studied by analyzing the residual concentration of the two organophosphates as a function of time. The residue of organophosphates in MSM with diazinon and chlorpyrifos was affected by bacterial strain, organophosphate concentration, incubation time, and their interactions (Table 3).
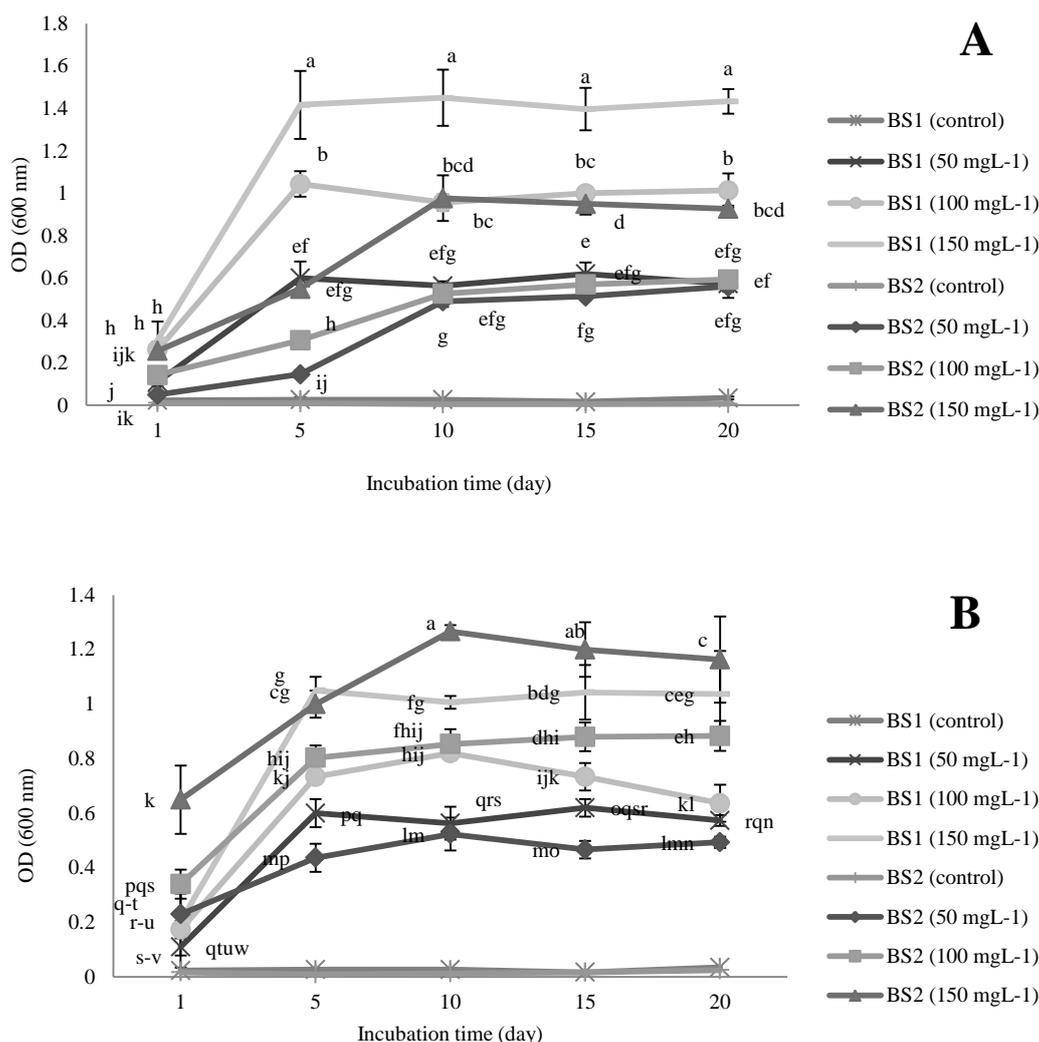
Fig. 1- Duncan's multiple range tests of means of OD_{600nm} in MSM as affected by bacterial strain and time of incubation in two media with diazinon (a) and chlorpyrifos (b) in three concentrations of organophosphate.
* Means followed by the same letter are not significantly different (p<0.05).

Table 3- Analysis of variance (mean square) of the effect of bacterial strain (BS), organophosphate concentration (OC), incubation time (IT) and their interactions on organophosphate residue in the MSM with diazinon and chlorpyrifos

Source of variation	diazinon		chlorpyrifos	
	Df	Mean square	Df	Mean square
BS	2	27339.20**	2	25515.90**
OC	2	33513.00**	2	35832.20**
BS*OC	4	3200.70**	4	2968.60**
Error	18	5.80	18	30.10**
IT	3	19803.00**	3	18389.40**
BS*IT	6	4211.60**	6	3661.30**
OC*IT	6	2128.70**	6	1831.80**
BS*OC*IT	12	600.60**	12	495.10**
Error	54	6.50**	54	9.40**

** Mean square of the treatment is significant at the 0.01 level

As seen (Fig. 2), increasing incubation time resulted to significantly decreasing concentration of pesticide in the MSM in inoculated media compared to control (un-inoculated with bacteria) in all the three concentration. On average, in three concentrations, the remaining of diazinon after 20 days of incubation in BS1 (11.73 mgL^{-1}) culture was significantly lower than that of the other strain (17.55 mgL^{-1}). So they were able to utilize diazinon as carbon substrate source, and BS1 isolate degraded diazinon more efficiently (Figure 2, (a)).

The isolates were also able to utilize chlorpyrifos as a sole carbon source (Figure 2 (b)). In a medium containing chlorpyrifos in each concentration, there was no significant difference between residues of chlorpyrifos in two strains of bacterial medium in the 4th day of incubation. But, on average, the remaining of chlorpyrifos in BS2 (11.65 mgL^{-1}) culture was significantly lower than that of the other strain (18.93 mgL^{-1}) after 20 days of incubation in a way that the least amount of chlorpyrifos belonging to the 20th day of incubation time in BS2 culture. So BS2 degraded chlorpyrifos more effectively than BS1.
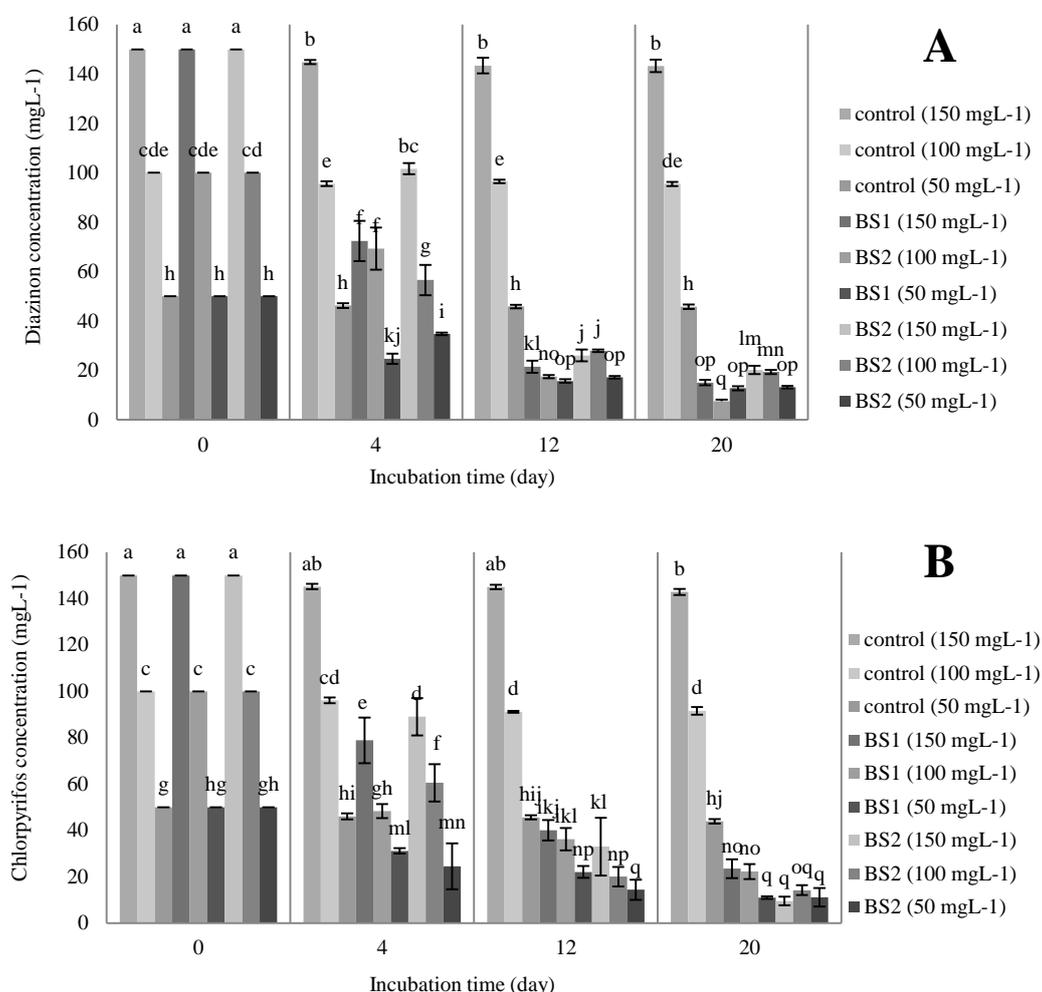
Fig. 2- Duncan's multiple range tests of means of organophosphate residue (mgL^{-1}) in MSM as affected by bacterial strain and incubation time in two media with diazinon (a) and chlorpyrifos (b) in three concentrations of organophosphate.

* Means followed by the same letter are not significantly different (p<0.05).

Statistical analysis showed that the bacterial strain (BS), the initial concentration of organophosphate (OC) and their interaction had significant effects on the degradation constant (Table 4). The means of the degradation rate (day^{-1}) of diazinon and chlorpyrifos by the isolated bacteria in MSM during 20 days is presented in Table 5 for each concentration. As seen, degradation constant (K day^{-1}) by both of bacterial strains was more than that for control treatment (Table 5). Analysis of the degradation constant (K day^{-1}) demonstrated that the degradation rate was increased by increasing concentration of organophosphate from 50 to 150 mgL^{-1}. Degradation constant in all three concentrations in MSM with diazinon inoculated with BS1 was more than that inoculated with BS2. In the case of chlorpyrifos, there was a reverse trend and BS2 was more efficient than BS1.

Table 4- Analysis of variance (mean square) of the effect of bacterial strain (BS) and organophosphate concentration (OC) on the degradation rate (k, day $^{-1}$) in the MSM with diazinon and chlorpyrifos as a source of carbon

Source of variation	diazinon		chlorpyrifos	
	Df	Mean square	Df	Mean square
BS	2	0.286**	2	0.264**
OC	2	0.012**	2	0.006**
BS*OC	4	0.004**	4	0.002*
Error	18	0.0001	18	0.00087

*, ** Mean square of the treatment is significant at the 0.05 and 0.01 level, respectively.

Table 5- Duncan's new multiple range tests of means of degradation rate (K day-1) in MSM as affected by bacterial strain (BS) and diazinon and chlorpyrifos concentration

Bacterial strain	Concentration mgL^{-1}	Diazinon degradation rate (day^{-1})	Chlorpyrifos degradation rate (day^{-1})
BS 1	50	0.164d	0.137c
	100	0.212b	0.170bc
	150	0.238a	0.175b
BS 2	50	0.130e	0.166b
	100	0.169d	0.191b
	150	0.189d	0.224a
control	50	0.014f	0.016d
	100	0.007f	0.011d
	150	0.007f	0.006d

* Means followed by the same letter in each column are not significantly different (p<0.05)

The half-life of the organophosphates was also estimated. The half-life (DT$_{50}$) of diazinon by isolated bacteria from Hamadan (BS1) and Damavand (BS2) were found to be 3.38 and 4.26 days, respectively. For chlorpyrifos, they were 4.31 and 3.57 days, respectively. The half-life of the two organophosphates by the two strains was different and significantly far lower than that in control.

Identification and characterization of degrading bacteria: Characteristic BS1 of the isolated colonies on the plate medium was cream-colored, and the surfaces of BS1 colonies were glossy with clear margins. The other (BS2) had the same characteristics, except that the colonies were brilliant green.

The microbial cultures were analyzed using Fatty Acid Methyl Esters (FAME). Figure 3 shows peaks corresponding to the fatty acids identified through FAME analysis of BS1 and BS2. The MIDI Sherlock microbial identification system identified the organisms to be *Acinetobacter olivarius* and *Pseudomonas aeruginosa* with 0.869 and 0.704 similarity index (SIM), respectively.

The 16S rRNA gene sequence of the fast growing and better isolates in degradation of diazinon (BS1) was also studied. From the genomic DNA of the isolated bacterium, 16S rRNA was amplified by Polymerase Chain Reaction (PCR). 1,525 bp-sized 16S rRNA was amplified (Figure 4).

The sequence analysis also showed similar results. After the completion of BLAST analysis, the organism (BS1) was identified to *Acinetobacter olivarius* strain with %99 identity match.

Analyses of biochemical patterns by API 20 NE test systems also supported the good identification of the isolates. Detailed analysis of biochemical properties of two isolates allowed differentiating and identifying BS1 and BS2 as *Acinetobacter olivarius* and *Pseudomonas aeruginosa* with 77.9% and 85.4% ID, respectively.

Sim Index	Entry Name
0.869	*Acinetobacter olivarius*

Sim Index	Entry Name
0.704	*Pseudomonas aeruginosa*
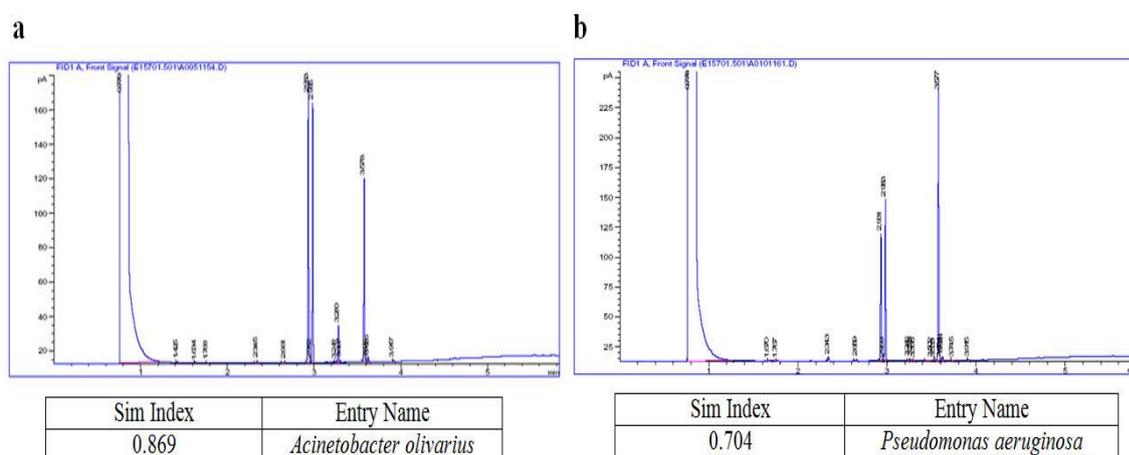
Fig. 3- Chromatogram of bacterial samples strain1 (BS1) (a) and strain 2 (BS1) (b) showing the fatty acid peaks.
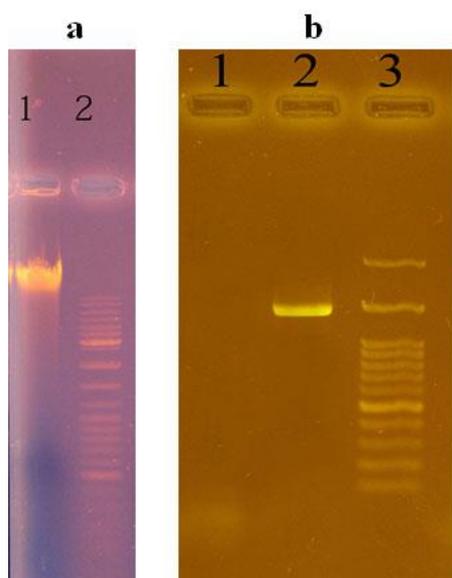
Fig. 4- PCR amplification of the 16S rRNA gene of the bacterium. a) Purification of genomic DNA. Lane 1: purified genome, Lane 2: DNA ladder mix. b) Amplification of the 16S rRNA gene. lane1: negative control, lane 2: PCR product with expected size (approx. 1.5 kb), lane 3: 100 bp DNA Ladder.

Discussion

The use of high concentration of organophosphates is a common way to isolate degrading bacteria (21, 22). For this purpose, the bacterial populations of the two soils were cultured in MSM liquid medium supplemented with 50 mgL^{-1} diazinon. Then the ability of these bacteria to degrade chlorpyrifos was also evaluated. The bacteria sub-cultured to obtain pure cultures before transferring them to biodegradation media with concentrations of 50, 100, 150 mgL^{-1} diazinon and chlorpyrifos.

Analysis of OD$_{600nm}$ in different incubation times showed that *A. olivarius* (BS1) during the 5th days of incubation reached the highest OD$_{600nm}$ value, while *P. aeruginosa* (BS2) achieved maximum OD$_{600nm}$ at 9th days of incubation in MSM medium. So *A. olivarius* grew faster than *P. aeruginosa*.

In terms of the degradation capacity, both isolates were able to degrade

chlorpyrifos and diazinon in MSM media under controlled culture conditions indicating their capacity to utilize diazinon and chlorpyrifos as a carbon source.

Results of the degradation rate in different concentrations of each insecticide revealed that increasing concentrations of insecticides increased the rate of degradation. This reflects the ability of these bacteria to use and degrade the organophosphates at high concentration level.

The result also showed, on average, in three concentrations of organophosphate *A. olivarius* (BS1) and *P. aeruginosa* (BS2) degraded 88.27 % and 81.07% of the initial dose of diazinon and degraded 82.45% and 88.35% of the initial dose of chlorpyrifos as carbon source during 20 day of incubation, respectively. Therefore, in MSM with diazinon as the sole carbon source the degradation ability of *A. olivarius* was more than that of *P. aeruginosa* and considering the result of OD_{600nm} maybe the effectiveness of this bacteria in MSM containing diazinon was better than *P. aeruginosa* .The higher OD_{600nm} of *P. aeruginosa* in MSM medium reflected that this strain was more effectively able to degrade chlorpyrifos than *A. olivarius.*

Although chlorpyrifos has been reported to be resistant to degradation, chlorpyrifos-degrading microbial species have been already isolated from soil (23), which utilize chlorpyrifos as a carbon source via an enrichment procedure (16, 24). These microbes have the ability to use the insecticide as an energy source under high concentration of chlorpyrifos. It was reported that a mixed bacterial culture can utilize chlorpyrifos as a carbon source (25) and as a limited nitrogen source by *Phanerochaete chrysosporium* (26).

In this regard, several bacterial species can also use diazinon as a sole carbon source such as *Flavobacterium sp.* (8, 26) and *Agrobacterium sp.* (27, 28).

Same bacterial species may also participate in the biotransformation of other organophosphate insecticides (29). Some organophosphate insecticides such as diazinon, chlorpyrifos, ethion, parathion, fonofos, malathion and gusathion are degraded by microbial hydrolysis and used as carbon sources for instant pure and mixed cultures of *Arthrobacter sp.*, *Pseudomonas sp.* and *Flavobacterium sp.* (8, 30, 31).

Pathway degradation of organophosphate pesticide seems to be similar to the most of the studied bacteria. It seems that the *opd* gene, encoding organophosphate hydrolase enzymes involved in this process are located on plasmids and homologous of it are on the chromosome (32).

Clearly, an effective bioremediation technology based on such isolates requires further research investigating factors affecting their biodegradation rate specially in field condition which many factors affect the activity of these microorganisms and availability of the organophosphate in soil. Also, the potential of these two strains for the analysis of other organophosphate also needs to be investigated. Hence, the present study concludes that *A. olivarius* and *P. aeruginosa* can be used as a potential candidate for degradation of organophosphates from contaminated sites.

Discussion and conclusion

Excessive use of pesticides causes environment hazards, declined biodiversity and water pollution which leads to the disruption of the ecosystem. An enrichment procedure allowed isolating two bacterial strains identified as *Acinetobacter olivarius* and *Pseudomonas aeruginosa* that may participate in the efficient degradation of the organophosphate insecticides including diazinon and chlorpyrifos.

Results of the present study suggest that the bacterial species, which have been isolated, can be used to remediate the polluted area. However, the use of

pesticide-degrading bacteria requires an understanding of the ecology of bacteria in new contaminated environments.

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تجزیه زیستی ارگانوفسفره‌های دیازینون و کلروپیروفوس به وسیله
دو باکتری جداسازی شده از خاک‌های آلوده کشاورزی

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چکیده

مقدمه: حشره کش‌های ارگانوفسفره نظیر کلروپیروفوس و دیازینون به طور گسترده‌ای برای کنترل حشرات خـانگی، کشاورزی و امور دامپزشکی به کار می رود. این ترکیبات یک تهدید برای سلامتی عمومی و محیط زیسـت بـه شـمار می‌روند. از طرف دیگر نرخ تجزیه پایینی دارند و بنابراین می‌توانند برای مدت طولانی در خاک باقی بمانند. یکی از مهمترین فاکتورهایی که سرنوشت آفت کش های ارگانوفسفره را در خاک و آب تعیین می کند تجزیه زیستی است.

مواد و روش‌ها: دو باکتری تجزیه کننده دیازینون و کلروپیروفوس از خاک‌های آلوده به آفت کش‌ها جـدا سـازی شد. گونه باکتری‌های جدا شده با استفاده از *16S RNA* ریبوزومی و آنالیز متیله کردن اسید های چرب شناسایی شدند.

نتایج: همبستگی مثبت بین رشد میکروبی و تجزیه دو ارگانوفسفات مشاهده شد. به طور متوسط گونـه بـاکتری ۱ و ۲ به ترتیب ۸۸/۲۷ و ۸۲/۴۵ درصد از غلظت اولیه دیازینون و ۸۱/۰۷ و ۸۸/۳۵ درصد از غلظت ابتدایی کلروپیروفوس را در محیط کشت نمکی در طول ۲۰ روز گرما گذاری تجزیه کردند. گونه هـای بـاکتری‌های جداسازی شـده بـا عنـوان *Acinetobacter sp* و *Pseudomonas* شناسـایی شـدند. بـالاترین میـزان تجزیـه دیازینـون توسـط بـاکتری *Acinetobacter* و بالاترین مقدار تجزیه کلروپیروفوس توسط *Pseudomonas* در محیط های کشـت نمکـی مشـاهده شد.

بحث و نتیجه‌گیری: جدایه‌های خالص باکتری‌ها از این دو سم به عنوان منبع کربن استفاده کردند. ایـن بـاکتری‌هـا بیشتر ترکیبات سمی را در طول ۲۰ روز تجزیه کردند. بنابراین باکتری‌های جداسازی شده پتانسیل زیست پالایی را در خاک های آلوده به دیازینون و کلروپیروفوس دارند.

واژه‌های کلیدی: تجزیه میکروبی، زیست پالایی، کلروپیروفوس، دیازینون