Original Article

Isolation and Characterization of Diazinon Degrading Bacteria from Contaminated Agriculture Soils

*Mehdi Hassanshahian**

Received: 20.12.2015 *Accepted: 03.02.2016*

ABSTRACT

Background: Organic agricultural pesticides are so stable in ecosystems and biosphere. These compounds affect soil conditions and agricultural products. The aim of this study was isolation of diazinon-degrading bacteria from Kerman pistachio orchards, Southeastern Iran.

Methods: Diazinon-degrading bacteria were enriched in Bushnell-Hass medium. Identification and sequencing of prevalent degrading strains was performed using PCR based on amplifying of 16s rDNA.

Results: The soil of pistachio orchard has some degrading bacteria that are suitable for elimination of diazinon from soil and environment. Three diazinon-degrading bacteria strains belong to: *Pseudomonas putida* strain D*3, P. fluorescens strain* D1 and *Achromobacter piechaudii* strain D8. The best degrading strain (D1), up to 100 ppm, illustrated a good growth, whereas more than this concentration, the growth was reduced. The results of Gas-Chromatography (GC) confirmed the decomposition of organic pesticide by degrading-bacteria.

Conclusion: The results of Gas-Chromatography (GC) confirmed the decomposition of organic pesticide by degrading-bacteria.

Using these strains and other biological reclamation methods we can eliminate bio-environmental problems.

Keywords: Agricultural Pesticide, Degrading Bacteria, Diazinon.

IJT 2016 (4):13-20

INTRODUCTION

Over the past fifty years, pesticides have been essential in the agricultural world. Agriculture development and the variety of pests were the most important factors in increasing the use of pesticides around the world, especially in the developing countries [1]. Accumulation of pesticides in food, water, earth, and air is one of the controversy issues on the environment and human health risk. Long-term presence of chemical pesticides in soil leads to accumulation of it in the edible vegetables and proteins [2]. Through the soil erosion, particles of pesticides can get into the water. The effective presence of pesticides in moist soils is varied from a week to several years [2].

Diazinon is an organophosphate insecticide with broad spectrum. In normal circumstances, diazinon is a yellow-brown colored liquid with a distinctive odor. If it heated, it will produce toxic gases. This pesticide is entered into the market with different formulations. Due to its low solubility in water, its infiltration into the groundwater is too low, but relatively, has a high binding potential to soil; furthermore, depending on aeration it remains in the soil, even in water, weeks to months. Trivial amounts of it evaporate and decompose in the atmosphere within a few days to a few weeks but its global ecological effects cannot be ignored. Diazinon's emulsion has high stability in hard water [3]. It has the ability to accumulate in aquatic organisms.

There are about 53 to 60 pesticides and around 9 to 11 diseases within pistachio orchards and these could cause, randomly, between 40% to 100% damage and disease in pistachio orchards. Diazinon is one of the most important phosphoric pesticides, used in pistachio orchards. Making indiscriminate use of pesticides and non-compliance with the amount and concentration of it leads to loss and reduction of the number of natural enemies [4]. Lack of natural enemies will lead to a drastically increase in the pests;

1. Department of Biology, Shahid Bahonar University of Kerman, Kerman, Iran.
*Correspondence Author: E-mail: mshahi@uk.ac.ir

consequently, the usage of agricultural pesticides and environmental pollution will increase [5].

Pesticides are removed from the soli by chemical, physical, and biological factors; although, a large part of pesticides degrades by chemical and physical factors, some of them degrade very rapidly by microorganisms while some are resistant to microbial degradation and hard to eliminate. Different groups of microorganisms, including fungi and bacteria are involved in the degradation of agricultural pesticides. Bacteria of the genus *Pseudomonas*, *Flavobacterium*, *Alcaligenes*, and *Rhodococcus* are involved in metabolization of diazinon [6]. Many soil microorganisms are able to converting the diazinon into the non-toxic compounds, this process called "biodegradation". In this process, the carbon source and energy are provided by pesticides. Considering the role of pesticides in the development of modern agriculture and their effects on ecosystems, it seems, microorganisms are the best option for metabolization and elimination of them to improve soil conditions, and the quality of life in the ecosystem. Biodegradation by bacteria is the main way to remove diazinon and other organophosphate pesticides from the environment [2]. Diazinin hydrolysis is the most important mechanisms for removal of this pesticide from the soil and water under aerobic and anaerobic conditions. The hydrolysis accelerates in acidic conditions. Two ways for degradation of diazinon were detected:

1. Degradation of diazinon to diazoxon and IMPH; knowing that diazoxon's toxicity is greater than IMPH. If diazoxon hydrolysis continues, it turns to DEP and IMPH.
2. In the second way, it decomposes to diethylthiophosphate and IMPH [2].

The purpose of this study was to investigate the existence of degrading bacteria in agricultural pesticide, diazinon, in Kerman pistachio orchards. After isolation the bacteria, the target is to measure the biodegradation ability of bacteria in order to remove this agricultural pesticide.

MATERIALS AND METHODS

Sampling

For isolation of diazinon-degrading bacteria sampling was performed in sterile conditions from different pistachio orchards from Kerman, southeastern Iran. Using sterile gloves the upper layer of soil (dirt and straw) was pushed away, then soil samples were obtained from 0-10 cm depth and were poured into the sterile plastic bags, the pocket doors were closed well, and transported on ice to the laboratory. Soil samples were placed in the refrigerator at 4°C until further analysis.

Isolation of Diazinon's Degrading Bacteria

For isolation the diazinon-degrading bacteria from agricultural soil, the sterilized Bushnell– Hass medium (BH) was used by adding a portion of the pesticide which was ultimately 50 ppm. Bushnell-Hass medium composition is as follows (grams per liter): 1 gr KH_2PO_4, 1gr K_2HPO_4, 0.2 gr $MgSO_4.7H_2O$, 0.02 gr $CaCl_2$, 1 gr NH_4NO_3, and two drops of $FeCl_3$ 60% , the pH was 7. Five grams of soil samples was inoculated for primary enrichment and placed into shaking incubator (180 rpm, INFORS AG) at 30 °C, Erlenmeyer flasks were covered with aluminum foil to prevent the optical degradation of pesticide. After 12 days, they were transferred from flasks into a new medium (BH). It was repeated for 3 times. Then, from the last passage, 100 µl of each sample, was inoculated on nutrient agar medium (NA). Various bacteria colonies were isolated and were cultured on toxin- agar medium [7].

Screening and Selection of the Best Diazinon-Degrading Bacteria Strains

In order to screen the most efficient diazinon-degrading bacteria sterilized BH medium with eventually concentration of diazinon (50 ppm), was used. Flasks were covered with aluminum foil to prevent optical degradation of pesticide. After 12 days serial dilutions methods was used for counting of bacteria. In this method, serial dilutions were made from 10^{-1} to 10^{-6}. After that from the three last dilutions, separately, 100 µl was poured into the plates by micropipette, and molten sterile count agar was added to it at temperature about 45 °C and streak culture was performed. Plates were incubated for 24 h at 30 °C, finally the colony of different dilutions was counted, which were from 20 to 200, and counts of bacteria in each dilution were calculated. By comparison, between the same dilutions in pesticide, the best strains were chosen and screened considering the number of colonies [1].

Peruse of the Capability of Bacteria by Using Diazinon for Growth

To study the ability of bacteria for using of diazinon as sole carbon source, purified cultures of isolated bacteria from the BH medium were transferred into nutrient agar medium. Then each pure bacterium was inoculated on toxin- agar medium and was incubated at 30 ºC for 7 days.

The Effect of Different Concentrations of Diazinon on the Growth of Prevalent Strains

After the screening of prevalent diazinon-degrading strains, the effect of four different concentrations of diazinon, including (30, 50, 70,100 ppm), on growth of prevalente starins was investigated. Flasks were covered with aluminum foil to prevent the optical degradation of pesticide, and placed on shaker (180 rpm) at 30 ºC for 12 days. The growth of bacteria in different concentartion of diazinon were estimated indirectly by serial dillution and pour plate methods as described in section 2-3 [6].

Identification of Isolated Bacteria

Biochemical Characterization

To identify and characterize the bacteria isolates, biochemical tests such as Gram staining, TSI, Simon's citrate, SIM, Urease, Oxidase, Catalase, MR-VP, Urea hydrolysis, oxidase, Oxidation - Fermentation of sugars (OF), and starch hydrolysis test were performed according to Bergey's Manual of Systematic Bacteriology (taxonomy) [8].

Molecular Identification

Analysis of the 16S rRNA gene was performed for the taxonomic characterization of the isolated strains. Total DNA was extracted from the bacterial strains using the CTAB method [9]. The bacterial 16S rRNA loci were amplified using the domain- specific forward primer Bac27_F (5-AGAGTTTGATCCTGGCTCAG-3) and the universal reverse primer Uni_1492R (5-TACGYTACCTTGTTACGACTT-3). The amplification reaction was performed in a total volume of 50 µl containing 1X solution Q (Qiagen, Hilden, Germany), 1X Qiagen reaction buffer, 1 µM of each forward and reverse primer, 10 µM dNTPs (Gibco, Invitrogen Co., Carlsbad, CA), and 2 U of Qiagen Taq polymerase (Qiagen). Amplification for 35 cycles was performed in a GeneAmp5700 thermocycler (PE Applied Biosystems, Foster City, CA, USA). The temperature profile for PCR was 95 ^{o}C for 5 min(1 cycle); 94 ^{o}C for 1 min and 72 ^{o}C for 2 min (35 cycles); and 72 ^{o}C for 10 min after the final cycle [10]. The amplified 16S rRNA fragment was sequenced with a Big Dye Terminator v3.1 Cycle sequencing kit on an automated capillary sequencer (model 3100 Avant Genetic Analyzer, Applied Biosystems). The similarity rank from the Ribosomal Database Project RDP) [11] and FASTA Nucleotide Database Queries were used to estimate the degree of similarity to other 16S rRNA gene sequences. Phylogenetic analysis of the sequences was performed as previously described [12].

Analysis the Amount of Diazinon by Gas Chromatography (GC)

Sterilized BH medium was prepared with diazinon (50 ppm) as sole carbon source and energy. Bacterial strains (500 µl) with concentration of 0.5 McFarland was added to each flask, and placed in shaking incubator (180 rpm) at 30 ºC for 12 d. Then 10 ml of the medium was removed from the flask and deionized water was added to reach to 50 ml. Then twice, each time for a half an hour with 20ml of n-hexane was shaked. Next, by using a funnel, the organic phase separated from the liquid phase and extracted by anhydrous sodium sulfate. Using the rotary at 45 ºC, it was dried and with 5 ml acetone it had reached to the volume and analyzed by GC . GC column was Hp-5 (30m × 0.32mm × 0.25µ), FID detector, which carrier gas was hydrogen. GC program was: the initial temperature at 60 ºC for one minute, the final temperature column was 270 ºC, injection temperature at 250 ºC, detector temperature 300 ºC, and the amount of flow was 2 µl [13].

RESULTS

Isolation and Characterization O Diazinon-Degrading Bacteria: Bacterial Characteristics

Ten strains of the diazinon-degrading bacteria in Kerman pistachio orchards were isolated. Some characteristics of these bacteria are shown in Table 1.

Table 1. Some Characteristics of isolated diazinon-degrading bacteria.

Strain	Morphology	Gram Staining	Citrate	Oxidase	Catalase	SIM	Urease	Gelatinase	Starch hydrolysis	MR	O/F
D1	Bacilli	-	+	+	+	-	-	+	-	+	+/-
D2	Bacilli	-	+	-	+	+	+	-	-	+	+/+
D3	Bacilli	-	+	+	+	+	+	-	-	+	+/-
D4	Diplobacilli	-	+	+	+	+	+	-	+	+	+/-
D5	Coccobacilli	-	+	-	+	+	+	-	-	+	+/+
D6	Vibrio	-	+	+	+	-	-	-	-	+	+/+
D7	Coccobacillii	-	+	-	+	+	-	-	+	+	+/-
D8	Bacilli	-	+	+	+	+	+	-	-	-	+/-
D9	Coccus	+	-	+	+	-	-	+	-	+	+/-
D10	Diplo Coccus	+	-	+	+	+	+	-	-	+	+/+

Selection of Predominat Diazinon-Degrading Strains

Due to the growth characteristics of isolated bacteria in media containing organic agricultural pesticides (diazinon) as the sole source of carbon and energy, some strains, which had low growth in this condition, were excluded. Other screening criteria for selection of premiere strains for degradation of organic pesticides were morphological and physiological similarities in analogous strains. In general, in a multi-stage screening, strains D1, D3, and D8 were chosen for the next steps. As a result, from the 10 isolated strains 3 of them were chosen as premiere strains (Table 1).

Molecular Identification of Predominant Diazinon-Degrading Strains

Molecular identification of bacteria with superior degradation of organic agricultural pesticides was conducted by partial amplification of 16 S rDNA. Then 1400bp, which is the product of PCR, was extracted from gel and was purified and sequenced. The sequences were blasted in GeneBank and the highest homology (above 98%) as genera and species of bacteria were determined. The results of the identification procedure showed that three isolated bacteria belong to *Pseudomonas fluorescens* (Strain D1), *P. putida* (Strain D3) and *Achromobacter piechaudii* (Strain D8). All sequences of three bacteria were submitted to the Genetic Sequence Database at the National Center for Biotechnical Information (NCBI). The gene bank IDs of these strains in NCBI are HF572848 (for D1), HF572849 (for D3) and HF572852 (for D8). The phylogenic trees of these two isolated strains were illustrated in Figure 1 and 2.

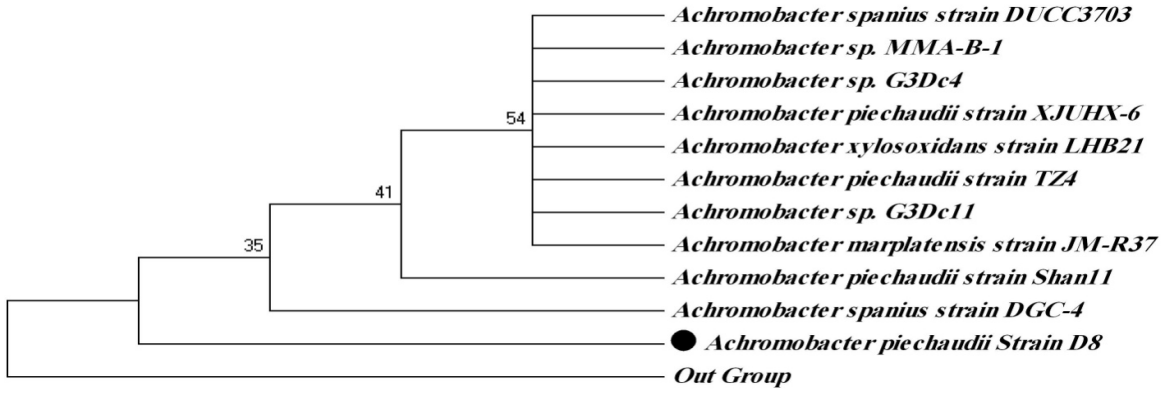

Figure 1. Phylogenetic tree of 16S rRNA sequences from the bacteria isolates strain D8. The tree was constructed using sequences of comparable regions of the 16S rRNA gene sequences that are available in public databases.

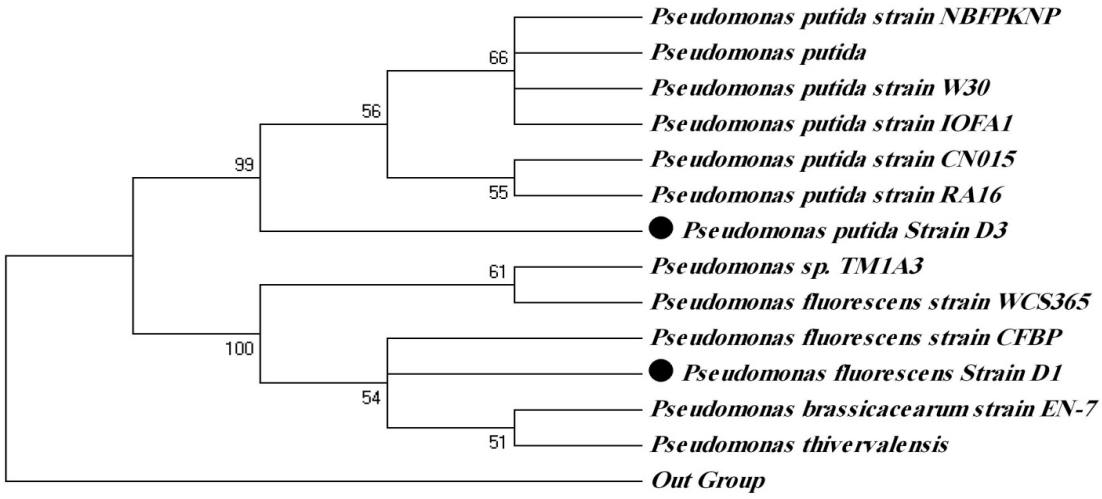

Figure 2. Phylogenetic tree of 16S rRNA sequences from the bacteria isolates strains D1 and D8. The tree was constructed using sequences of comparable regions of the 16S rRNA gene sequences that are available in public databases.

The Rate of Diazinon Degradation by Isolated Bacteria

The percentage degradation of diazinon was calculated by comparing the gas chromatograms of the undegraded controls with those from the tests for each strain (Figure 3). Three strains can degraded the majority portion of diazinon after 15 d. The percentage of degradation for isolated strains was as follows: Strain D1 (95 %), strain D3 (62 %) and strain D8 (85 %). Then the best-isolated strain for biodegradation of diazinon was *P. fluorescens* strain D1.

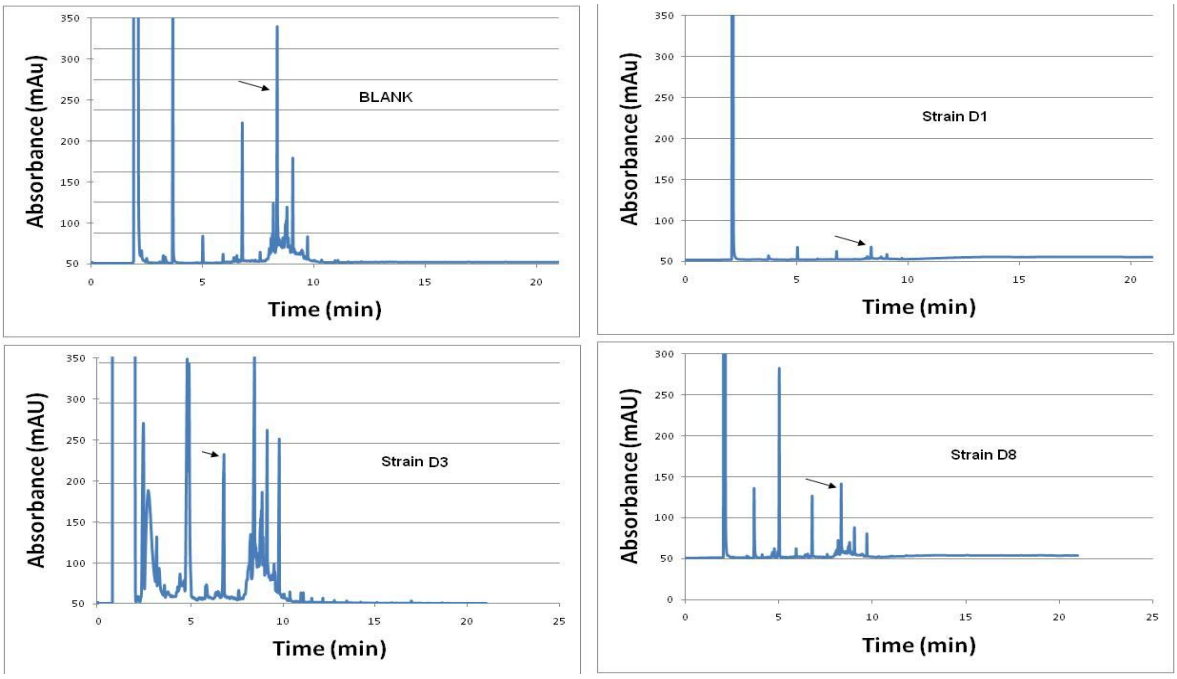

Figure 3. Gas Chromatography (GC) tracing of residual diazinon in the control flask and in the flasks incubated with degrading bacteria after 1 week of incubation at 30 ^{o}C and 165 rpm. The arrows show the diazinon peak.

The Effect of Different Concentrations of Diazinon on P. fluorescens Strain D1

The effect of different concentrations of diazinon on *P. fluorescens* strain D1 were tested by growth of this strain in increasing concentrations of diazinon from 30 to 100 ppm (Figure 4). When the concentration of diazinon increased the growth of strain D1 were decreased and the best concentration that degraded by this strain was 40 ppm.

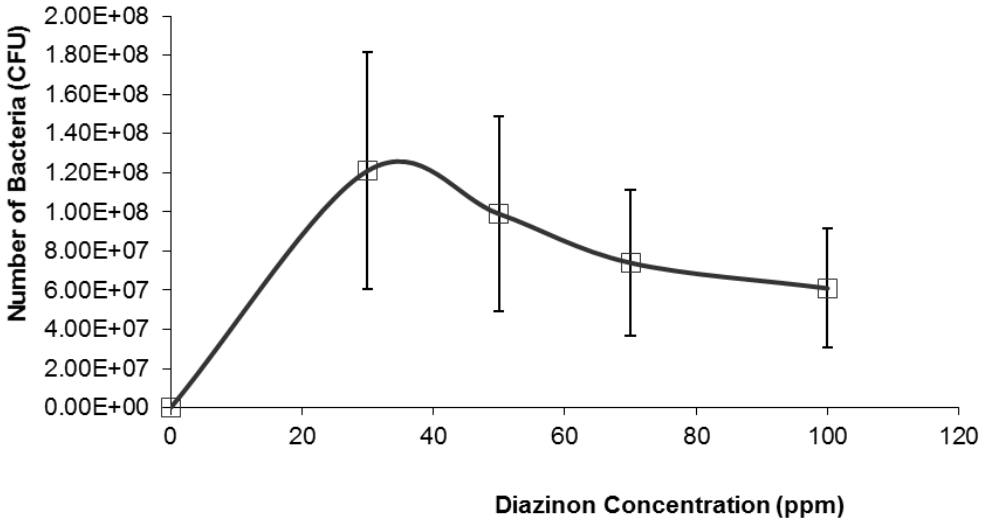

Figure 4. The effect of diazinon concentrations on the growth of strain D1.

DISCUSSION

So far, degrading bacteria of organic agriculture pesticide have been examined by researchers in different environments and terrestrial ecosystems. Various species are able to degrade agricultural organic pesticides [3]. The wide usage of organophosphate pesticides throughout the world, a rise in the awareness of sustainability and their toxicity and irreparable effects on humans and ecosystems of the earth, cause a special consideration on microbial degradation [2]. In 1973, biodegradation of organophosphate pesticides by plants, soil and animals was reported. In 1979, the main factor of degradation of the organophosphate insecticides was reported as microbial cleavage [14-16]. In 2001, Bhadbade through the wide examination of microbial degradation in 10 soil samples in India could isolate 22 strains of bacteria belonging to the *Stomatocucus, Planococcus, Pseudomonas, Arthrobacter* and *Bacillus* genus from soils, which had been exposed to organophosphate for a long period. Dshpande et al., isolated 26 strains of degrading Dimethoate organophosphate insecticides from floodplain soils, cow manure, and industrial sludge, which with determination of 16 S rRNA sequence, found that their genus as *Pseudomonas, Bacillus, Brevundimonas*, and *Klebsiella* [5]. Kanekar et al., investigated the Indian agricultural soils, which for many years had been exposed to these pesticides, and identified bacterial degrading pesticides for various pesticides including *Flavobacterium* sp. *and Pseudomonas* sp.; also, genetic and their enzymatic pathways were studied.

In this study, conventional organic agricultural pesticide, diazinon, and terrestrial ecosystems (pistachio orchards) contaminated with pesticides were used as segregation sources [6, 17]. Actually, the soil has been exposed to these pollutants for a long time. Selected ecosystem in this study is compatible with many researchers choice because according to the matching principle the degrading-bacteria are found in places that have been exposed to contamination. In this study, these principles were used to find the bacteria. Ten pesticides-degrading bacteria for diazinon were isolated. After screening, three predominate strains were chosen, which could show higher growth near these

pesticides. These three strains included *P. fluorescens strain* D1, *P. putida strain* D3 and *A. piechaudii strain* D8. An Increase in concentration of diazinon on the identified premiere bacteria will decrease its growth until 100-ppm concentration. This result could be interpreted such that, bacteria till 100-ppm concentration is correspondence with respect to degradation and uses carbon and energy source also, higher concentration more than 100 ppm is toxic to them.

So far, different methods have been used to investigate the amount of agriculture pesticide biodegradation. We can refer to Bioassay, Thin-layer chromatography, Gas chromatography, and GC-Mass as some of prevailing methods. [18, 19].

Cycon et al. using Gas Chromatography with TSD detector examined and confirmed the rate of degradation of diazinon by *Serratia* and *Pseudomonas* strains. Abo Amer et al. using Gas chromatography examined the rate of degradation of diazinon by *Serratia marcescens* [7,20]. In this study, Gas Chromatography with FID detector was used in order to evaluate diazinon degradation level. GC results showed that some strains had intensive reduction for peaks and in some cases degradation ability were medium or weak. GC results are in coincidence with obtained results from growth of bacteria in pesticides. Using these strains and other biological reclamation methods we can eliminate bio-environmental problems.

CONCLUSION

The diazinon degrading bacteria are widespread in agriculture soil of pistachio gardens at Kerman province. These bacteria may be useful for remediation of soil for toxic compounds.

ACKNOWLEDGEMENTS

This work was financially supported by grants from Shahid Bahonar University of Kerman.

REFERENCES

1. Drufovka K, Danevcic T, Trebs P, Stopar D. Microorganisms trigger chemical degradation of diazinon. Inter Biodeterior Biodegrad 2008; 62: 293–296.
2. Singh BK, Walker A. Microbial degradation of organophosphorus compounds. FEMS Microbiol Rev 2006; 30: 428–471.
3. Sanchez ME, Estrada IB, Martinez O, Martin-Villacorta J, Aller A, Moran A. Influence of the application of sewage sludge on the degradation of pesticides in the soil. Chemosphere 2004; 57: 673–679.
4. Yasouri FN. Plasmid mediated degradation of diazinon by three bacterial strains *Pseudomonas* sp., *Flavobacterium* sp. and *Agrobacterium* sp. Asian J Chem 2006; 18: 2437–2444.
5. Deshpande N. Biodegradation of Dimethoate-a carbamate group of organophosphorus insecticides. Lett Appl Microbiol 2002; 33: 275-79.
6. Kanekar P, Bhadbhade B. Biodegreadtion of Organophosphorus Pseticides. Proc Indian natn Sci Acad 2004; 70: 57-70.
7. Cycoń M, Wójcik M, Piotrowska Z. Biodegradation of the organophosphorus insecticide diazinon by *Serratia* sp. and *Pseudomonas* sp. and their use in bioremediation of contaminated soil. Chemosphere 2009**;** 76: 494-501.
8. Holt SG, Kriey NR, Sneath PHA, Staley JT, Williams ST. Bergy's manual of determinative for bacteriology 1998; Williams and Wilkins, New York.
9. Winnepenninckx B, Backeljau T, Dewachter R. Extraction of high molecular weight DNA from molluscs. Trends Genet 1993; 9: 407-411.
10. Maidak BL, Olsen GJ, Larsen N. The RDP Ribosomal Database Project. Nucleic Acids Res 1997; 25: 109-1 11.
11. Troussellier M, Got P, Mboup M, Corbin D, Giuliano L, Cappello S, Bouvy M. Daily bacterio plankton in a sub-Saharan estuary (Senegal River, West Africa): a mesocosm study. Aquat Microb Ecol 2005: 40: 13-24.
12. Yakimov MM, Cappello S, Crisafi E, Tursi A. Phylogenetic survey of metabolically active microbial communities associated with the deep-sea coral Lophelia pertusa from the Apulian Plateau, Central Mediterran Sea. Deep Sea Res part I (2006); 53: 62-75.
13. Abo A, Aly E. Biodegradation of Diazinon by Serratia marcescens DI101 & its use in bioremediation of contaminated Environment. J Microbial Biotecnol 2011; 21: 71-80.
14. Rosenberg A, Alexander M. Microbial cleavage of various Organophosphorus insecticides. Apple Environ Microbiol 1979; 37: 886-91.
15. Beynon K, Hutson D, Wright A. The metabolism & degradation of Vinyl phosphate insecticides. Res Rev 1973; 47: 55-142.
16. Bayat Z, Hassanshahian M, Askeri Hesni M. Enrichment and isolation of crude oil degrading bacteria from some mussels collected from the Persian Gulf. Mar Pollut Bull 2015; 101 (1): 85–91.
17. Emtiazi G, Saleh T, Hassanshahian M. The effect of bacterial glutathione S-transferase on

morpholine Degradation. Biotechnol J. 2009; 4: 202–5.
18. Hassanshahian M, Zeynalipour M.S, Hosseinzadeh Musa F. Isolation and characterization of crude oil degrading bacteria from the Persian Gulf (Khorramshahr provenance). Mar Pollut Bull 2014; 82: 39–44.
19. Hassanshahian M, Emtiazi G, Cappello S. Isolation and characterization of crude-oil-degrading bacteria from the Persian Gulf and the Caspian Sea. Mar Pollut Bull 2102; 64: 7–12.
20. Hassanshahian M. Isolation and characterization of biosurfactant producing bacteria from Persian Gulf (Bushehr provenance). Mar Pollut Bull 2014; 86: 361–6.