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## Isolation and characterization of endosulfan-degrading bacteria from contaminated agriculture soils

Mehdi Hassanshahian\*, Zahra Shahi

Department of Microbiology, Kerman Branch, Islamic Azad University, Kerman, Iran

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### ABSTRACT

**Objective:** To isolate and characterize endosulfan-degrading bacteria from Kerman pistachio orchards.

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

**Results:** The results showed that the soils of pistachio orchards have some degrading bacteria that are suitable for elimination of endosulfan from soils and the environment. Four endosulfan-degrading bacteria strains belong to *Achromobacter xylosoxidans* (strain EN3), *Pseudomonas azotoformans* (strain EN4), *Pseudomonas brassicacearum* (strain EN7) and *Pseudomonas thivervalensis* (strain EN8), respectively. The best degrading strain (EN7), up to 100 mg/L, illustrated a good growth, whereas the growth was reduced in concentration higher than 100 mg/L. The results of gas chromatography confirmed the decomposition of organic pesticide by degrading-bacteria.

**Conclusions:** By using these strains and other biological reclamation methods we can eliminate bio-environmental problems.

## 1. Introduction

The widespread use of potentially harmful pesticides has recently been under scrutiny in many parts of the world. The organochlorine pesticides are one of the major chemicals responsible for the contamination and deterioration of soils and water environments especially in agricultural fields[1].

During the past 50 years, pesticides have been the essential part of the agricultural world. Although it is evident that the production and distribution of pesticides increases the quality and efficiency of the agricultural industry, the pesticides is likely to be used improperly and unreasonably[2]. Despite their benefits, pesticides are compounds that may have toxic side effects causing potential environmental risk[3].

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine 3-oxide), an organochlorine insecticide, is a mixture of the two isomers,  $\alpha$ - and  $\beta$ -endosulfan.

It is a contact and stomach poison used to control chewing and sucking insects, such as Colorado beetle, flea beetle, cabbage worm aphids and leaf hopper. This insecticide is used to control insect pests of a wide range of crops, including cereals, cotton, coffee, fruits, oil seeds and vegetables[4].

In the close vicinity of agricultural fields, the contamination of atmosphere, soils, sediments, surface and rain waters, and foodstuffs by endosulfan has been documented in numerous previous studies[5]. The persistence of endosulfan in soil and water environments has been observed by different researchers under different conditions[6]. Its harmful impacts on aquatic fauna and numerous mammalian species including human beings have been reported several times in literature[7,8].

It has been reported that endosulfan is genotoxic in mammalian cells[5]. Due to the abundant usage and potential transport of endosulfan, contamination is frequently found in the environment at considerable distance from the point of application[9]. These health and environment concerns have led to an interest in degradation and detoxification of endosulfan. Detoxification of endosulfan through biological means is receiving serious attention as compared to existing methods such as incineration and landfill[10].

Biodegradation is an efficient bioremediation technique in microorganisms which grow in different ecosystems and through

\*Corresponding author: Mehdi Hassanshahian, Department of Microbiology, Kerman Branch, Islamic Azad University, Kerman, Iran.

Tel: + 98 343222067

Fax: + 98 34322022

E-mail: [hasanshahi@gmail.com](mailto:hasanshahi@gmail.com)

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symbiosis with xenobiotics. These microorganisms are able to survive even in incompatible conditions. Endosulfan has been used as a source of sulfur for microbial growth and as a carbon resource in bioremediation in various studies. Endosulfan is decomposed into endosulfan sulfate by oxidation and into endosulfan diol by hydrolysis. Endosulfan sulfate is also toxic and stable as the major component of endosulfan. Endosulfan diol can be converted to endosulfan ether, endosulfan hydroxyl ether, endosulfan dialdehyde, and endosulfan lactone. However, these metabolites are less toxic[11].

There are about 53 to 60 pesticides and around 9 to 11 diseases within pistachio orchards and these could cause randomly 40% to 100% damage and disease in pistachio orchards[12]. Endosulfan is one of the most important organochlorine insecticides, which is used in pistachio orchards. Endosulfan is the most widely used insecticide in Iran to control pests in pistachio gardens. These gardens are mostly located at Rafsanjan City in Kerman Province of Iran. The objective of the present study was to find out endosulfan-degrading bacteria and to study its phylogenetic relationships.

## 2. Materials and methods

### 2.1. Sampling

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

### 2.2. Isolation of endosulfan-degrading bacteria

As for isolation of endosulfan-degrading bacteria from agricultural soil, the sterilized Bushnell-Hass medium was used by adding a portion of the pesticide which was ultimately 50 mg/L. Bushnell-Hass medium composition was as follows (g/L): 1 g  $\text{KH}_2\text{PO}_4$ , 1 g  $\text{K}_2\text{HPO}_4$ , 0.2 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.02 g  $\text{CaCl}_2$ , 1 g  $\text{NH}_4\text{NO}_3$ , and two drops of  $\text{FeCl}_3$  (60%), and the pH was 7. Five grams of soil samples were inoculated for primary enrichment and placed into shaking incubator (180 r/min, 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 Bushnell-Hass medium. It was repeated for 3 times. Then, from the last passage, 100  $\mu\text{L}$  of each sample was inoculated on nutrient agar medium. Various bacteria colonies were isolated and were cultured on toxin-agar medium[13].

### 2.3. Screening and selection of the best endosulfan-degrading bacterial strains

In order to screen the most efficient endosulfan-degrading bacteria, sterilized Bushnell-Hass medium with endosulfan (50

mg/L) was used. Flasks were covered with aluminum foil to prevent optical degradation of pesticide. After 12 days, serial dilutions method was used for counting bacteria. In this method serial dilutions were made from  $10^{-1}$  to  $10^{-6}$ . After that, from the last three dilutions, separately, 100  $\mu\text{L}$  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 between 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[14].

### 2.4. Peruse of the capability of bacteria growing with endosulfan

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

### 2.5. The effect of different concentrations of endosulfan on the growth of prevalent strain

After the screening of prevalent endosulfan-degrading strains, the effect of four different concentrations of endosulfan, including 30–100 mg/L, on growth of prevalent strains was investigated. Flasks were covered with aluminum foil to prevent the optical degradation of pesticide, and placed on the shaker (180 r/min) at 30 °C for 12 days. The growth of bacteria in different concentrations of endosulfan was estimated indirectly by serial dilutions and pour plate methods[15].

### 2.6. Identification of isolated bacteria

#### 2.6.1 Biochemical characterization

To identify and characterize the bacteria isolates, biochemical tests such as Gram staining, triple sugar iron, Simon's citrate, sulfite, indol, motility, urease, oxidase, catalase, methyl red-voges proskauer, urea hydrolysis, oxidation-fermentation glucose, and starch hydrolysis tests were performed according to methods described by Holt *et al.*[16].

#### 2.6.2. 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 by using the hexadecyltrimethylammonium bromide method[17]. 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  $\mu\text{L}$  containing 1× solution Q (Qiagen, Hilden, Germany), 1× Qiagen reaction buffer,

1  $\mu\text{mol/L}$  of each forward and reverse primer, 10  $\mu\text{mol/L}$  dNTPs (Gibco, Invitrogen Co., Carlsbad, CA), and 2 IU of Qiagen *Taq* polymerase (Qiagen). Amplification for 35 cycles was performed in a GeneAmp 5700 thermocycler (PE Applied Biosystems, Foster City, CA, USA). The temperature for PCR was 95 °C for 5 min (1 cycle); 94 °C for 1 min and 72 °C for 2 min (35 cycles); and 72 °C for 10 min after the final cycle[18]. The amplified 16S rRNA fragment was sequenced with a BigDye Terminator v3.1 Cycle sequencing kit on an automated capillary sequencer (model 3100-Avant Genetic Analyzer, Applied Biosystems). The similarity ranked from the Ribosomal Database Project and FASTA Nucleotide Database Queries was used to estimate the degree of similarity to other 16S rRNA gene sequences. Phylogenetic analysis of the sequences was performed as previous description[18].

### 2.6.3. Analysis of the amount of endosulfan by gas-chromatography (GC)

Sterilized Bushnell-Hass medium was prepared with endosulfan (50 mg/L) as sole carbon source and energy. Bacterial strains (500  $\mu\text{L}$ ) with concentration of 0.5 McFarland were added to each flask, and placed in shaking incubator (180 r/min) at 30 °C for 12 days. Then 10 mL of the medium was removed from the flask and deionized water was added to reach to 50 mL. Then the sample was shaken twice with each time for a half an hour with 20 mL of *n*-hexane. Next, by using a funnel, the organic phase was separated from the liquid phase and extracted by anhydrous sodium sulfate. Using the rotary at 45 °C, it was dried and added with 5 mL acetone to reach to the volume and analyzed by GC. GC column was HP-5 column (30.00 m  $\times$  0.32 mm  $\times$  0.25  $\mu\text{m}$ ), with flame ionization detector, and carrier gas was hydrogen. GC program was as follows: the initial temperature at 60 °C for one minute, the final temperature of column was 270 °C, injection temperature 250 °C, detector temperature 300 °C, and the amount of flow was 2  $\mu\text{L}$ [19].

## 3. Results

### 3.1. Isolation and characterization of endosulfan-degrading bacteria: bacterial characteristics

In this study, 9 strains of the endosulfan-degrading bacteria in Kerman pistachio orchards were isolated. Some characteristics of these bacteria are shown in Table 1. As shown in this table, almost the isolated bacteria were Gram-negative, catalase and oxidase-positive.

**Table 1**

Some biochemical characteristics of endosulfan-degrading bacteria isolated.

Strain	Morphology	Gram staining	Citrate	Oxidase	Catalase	SIM	Urease	Gelatinase	Starch hydrolysis	O/F
EN1	Curved bacilli	-	+	-	+	--+	+	-	-	+/-
EN2	Cocccobacilli	-	+	+	-	--+	-	-	+	-/+
EN3	Bacilli	-	+	+	-	+++	-	-	+	-/+
EN4	Diplobacilli	-	+	+	-	--+	-	-	-	-/-
EN5	Cocccobacilli	-	+	-	+	---	+	-	-	+/-
EN6	Vibrio	+	+	-	+	--+	-	-	-	+/-
EN7	Bacilli	-	+	+	+	--+	+	-	-	-/+
EN8	Bacilli	-	+	+	+	---	-	-	-	-/+
EN9	Coccus	+	+	-	+	---	-	+	-	-/-

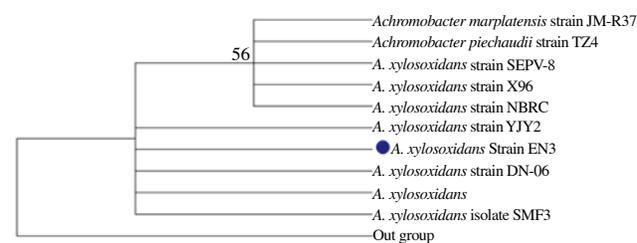
SIM: Sulfite, indol, motility; O/F: Oxidation/fermentation of sugars; +: Positive reaction; -: Negative reaction.

### 3.2. Selection of predominant endosulfan-degrading strains

Due to the growth characteristics of isolated bacteria in media containing organic agricultural pesticides (endosulfan) as the sole source of carbon and energy, some strains which had low growth under 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 EN3, EN4, EN7 and EN8 were chosen for further study. As a result, from the 9 isolated strains, 4 of them were chosen as premiere strains (Table 1).

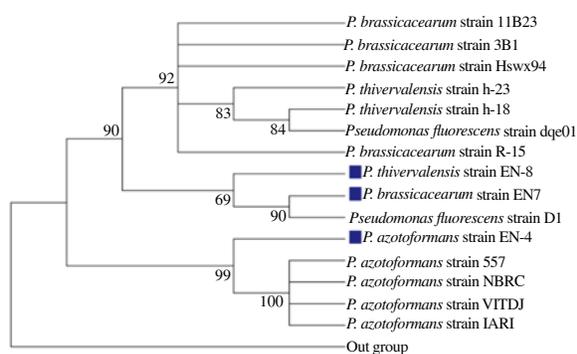
### 3.3. Molecular identification of predominant endosulfan-degrading strains

Molecular identification of bacteria with superior ability to degrade organic agricultural pesticides was conducted by partial amplification of 16S rDNA. Then the product of PCR (1400 bp), was extracted from gel, purified and sequenced. The sequences were blasted in GeneBank and the highest homology (above 98%) for genera and species of bacteria was determined. The results of the identification procedure showed that four isolated bacteria belong to *Achromobacter xylosoxidans* (*A. xylosoxidans*) (strain EN3), *Pseudomonas azotoformans* (*P. azotoformans*) (strain EN4), *Pseudomonas brassicacearum* (*P. brassicacearum*) (strain EN7), and *Pseudomonas thivervalensis* (*P. thivervalensis*) (strain EN8). All sequences of four bacteria were submitted to the Genetic Sequence Database at the National Center for Biotechnical Information. The gene bank IDs of these strains in the National Center for Biotechnical Information are HF572850 (for EN3), HF572854 (for EN4), HF572846 (for EN7) and HF572855 (for EN8). The phylogenetic trees of these four isolated strains were illustrated in Figures 1 and 2.



**Figure 1.** Phylogenetic tree of 16S rRNA sequences from the bacteria isolate strain EN3.

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 EN4, EN7 and EN8.

The tree was constructed using sequences of comparable regions of the 16S rRNA gene sequences that are available in public databases.

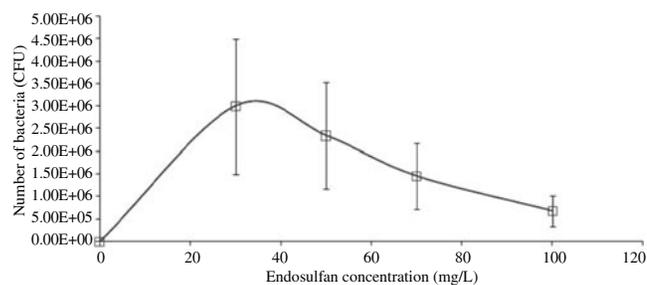
### 3.4. The endosulfan degradation by isolated bacteria

The percentage of degradation of endosulfan was calculated by comparing the gas chromatograms of the undegraded controls with those from the tests for each strain. The results were shown in Figure 3. The data obtained show that the four strains can degrade the major portion of endosulfan after 15 days. The percentages of degradation by isolated strains were as follows: 58% for strain EN3, 82% for strain EN4, 98% for strain EN7 and 54% for strain EN8. Then the best isolated strain for biodegradation of endosulfan was *P. brassicacearum* (strain EN7).

### 3.5. The effect of different concentrations of endosulfan on *P. brassicacearum* (strain EN7)

The effect of different concentrations of endosulfan on *P. brassicacearum* (strain EN7) was tested by the growth of this strain in increasing concentrations of endosulfan from 30 to 100 mg/L. The results were illustrated in Figure 4. As shown in this figure, when the concentration of endosulfan increased, the growth of

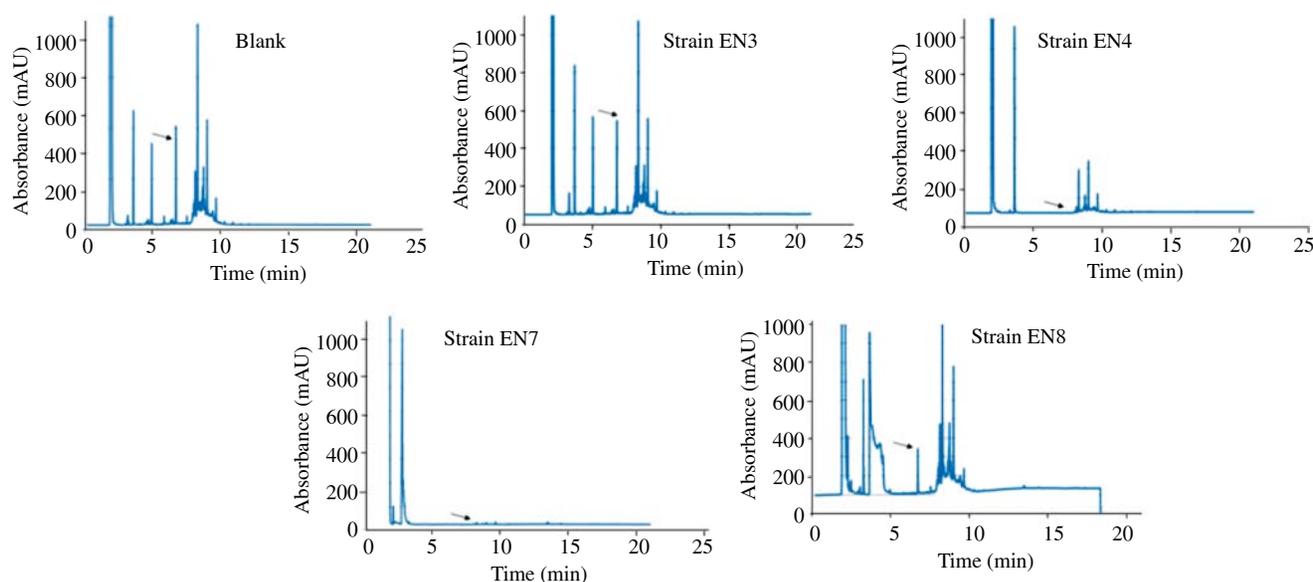
strain EN7 was decreased and the best concentration of endosulfan that degraded by this strain was 40 mg/L.



**Figure 4.** The effect of endosulfan concentrations on the growth of strain EN7.

## 4. Discussion

So far, organic agriculture pesticide-degrading bacteria have been examined by researchers in different environments and terrestrial ecosystems. Various species are able to degrade agricultural organic pesticides[20]. The wide usage of organochlorine pesticides throughout the world, a rise in the awareness of sustainability and their toxicity and irreparable effects on humans and the ecosystem of the earth cause a special consideration on microbial degradation[21]. In 1973, biodegradation of organochlorine pesticides by plants, soils and animals was reported by Beynon *et al.*[21]. In 1979, Rosenberg and Alexander recognized that the main factor of degradation of the organochlorine insecticides was microbial cleavage[12]. In 2001, Beynon through the wide examination of microbial degradation in 10 soil samples in India could isolate 22 strains of bacteria belonging to the *Stomatococcus*, *Planococcus*, *Pseudomonas*, *Arthrobacter* and *Bacillus* genus from soils which had been exposed to organochlorine for a long period of time[21]. Deshpande isolating 26 dimethoate organophosphate insecticides-degrading strains from floodplain soils, cow manure and industrial sludge, which with determination of 16S rRNA sequence found that they belonged to genus *Pseudomonas*, *Bacillus*,



**Figure 3.** GC tracing of residual endosulfan in the control flask and in the flasks incubated with degrading bacteria after 1 week of incubation at 30 °C and 180 r/min.

The arrows show the endosulfan peak.

*Brevundimonas*, and *Klebsiella*[22]. Kanekar *et al.* investigated the Indian agricultural soils in 2004, which had been exposed to these pesticides for many years, and identified pesticides-degrading bacteria for various pesticides including *Flavobacterium* sp. and *Pseudomonas* sp., and their genetic and enzymatic pathways were also studied[15]. In this study, conventional organic agricultural pesticide, endosulfan, and terrestrial ecosystems (pistachio orchards) contaminated with pesticides were used as segregation sources. Actually, the soil has been exposed to these pollutants for a long time. The selected ecosystem in this study is compatible with many researchers' choices because according to the matching principle the degrading bacteria are found in places which have been exposed to contaminations. In this study, these principles were used to find the bacteria. In this study, 9 pesticides-degrading bacteria for endosulfan were isolated. After screening, four predominant strains were chosen, which could show higher growth in the vicinity of this pesticide. These four strains belong to two genus: *Pseudomonas* and *Achromobacter*. An increase in the concentration of endosulfan will decrease the growth of identified premiere bacteria till 100 mg/L concentration. This result could be interpreted that, the highest concentration of endosulfan degraded by bacteria is 100 mg/mL. Bacteria can use this pollutant as energy and carbon source. Also, higher concentration more than 100 mg/L 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, GC, and GC-MS as some of prevailing methods.

Cycoń *et al.* examined and confirmed the rate of degradation of endosulfan by *Serratia* and *Pseudomonas* strains by using GC with thermionic specific detector in 2009[13]. Abo-Amer *et al.* examined the rate of degradation of endosulfan by *Serratia marcescens* using GC in 2010. In this study, GC with flame ionization detector was used to evaluate endosulfan degradation level[19]. GC results showed that some strains had intensive reduction in the amount of the 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. by using these strains and other biological reclamation methods we can eliminate bio-environmental problems in agriculture fields.

### Conflict of interest statement

We declare that we have no conflict of interest.

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### References

- Jayashree R, Vasudevan N. Effect of Tween 80 added to the soil on the degradation of endosulfan by *Pseudomonas aeruginosa*. *Int J Environ Sci Technol* 2007; **4**(2): 203-10.
- Oliveira-Silva JJ, Alves SR, Meyer A, Perez F, Sarcinelli PN, da Costa Mattos RC, et al. [Influence of socioeconomic factors on the pesticides poisoning, Brazil]. *Rev Saude Publica* 2001; **35**(2): 130-5. Portuguese.
- Golfinopoulos SK, Nikolaou AD, Kostopoulou MN, Xilourgidis NK, Vagi MC, Lekkas DT. Organochlorine pesticides in the surface waters of Northern Greece. *Chemosphere* 2003; **50**(4): 507-16.
- Shivaramaiah HM, Kennedy IR. Biodegradation of endosulfan by a soil bacterium. *J Environ Sci Health B* 2006; **41**(6): 895-905.
- Agency for Toxic Substances and Disease Registry. Toxicological profile for endosulfan. Atlanta: Agency for Toxic Substances and Disease Registry; 2000. [Online] Available from: <http://www.atsdr.cdc.gov/toxprofiles/tp.asp?id=609&tid=113> [Accessed on 20th October, 2015]
- Guerin TF, Kennedy IR. Distribution and dissipation of endosulfan and related cyclodienes in sterile aqueous systems: implications for studies on biodegradation. *J Agric Food Chem* 1992; **40**(11): 2315-23.
- Paul V, Balasubramaniam E. Effects of single and repeated administration of endosulfan on behavior and its interaction with centrally acting drugs in experimental animals: a mini review. *Environ Toxicol Pharmacol* 1997; **3**(2): 151-7.
- Chaudhuri K, Selvaraj S, Pal AK. Studies on the genotoxicity of endosulfan in bacterial systems. *Mutat Res* 1999; **439**(1): 63-7.
- Miles CJ, Pfeuffer RJ. Pesticides in canals of South Florida. *Arch Environ Contam Toxicol* 1997; **32**(4): 337-45.
- Siddique T, Okeke BC, Arshad M, Frankenberger WT Jr. Enrichment and isolation of endosulfan-degrading microorganisms. *J Environ Qual* 2003; **32**(1): 47-54.
- Castillo JM, Casas J, Romero E. Isolation of an endosulfan degrading bacterium from a coffee farm soil: persistence and inhibitory effect on its biological functions. *Sci Total Environ* 2011; **412-413**: 20-7.
- Rosenberg A, Alexander M. Microbial cleavage of various organophosphorus insecticides. *Appl Environ Microbiol* 1979; **37**(5): 886-91.
- Cycoń M, Wójcik M, Piotrowska-Seget Z. Biodegradation of the organophosphorus insecticide endosulfan by *Serratia* sp. and *Pseudomonas* sp. and their use in bioremediation of contaminated soil. *Chemosphere* 2009; **76**(4): 494-501.
- Drufovka K, Danevčič T, Trebše P, Stopar D. Microorganisms trigger chemical degradation of endosulfan. *Int Biodeterior Biodegr* 2008; **62**(3): 293-6.
- Kanekar PP, Bhadbhade BJ. Biodegradation of organophosphorus pesticides. *Proc Indian Nat Sci Acad* 2004; **70**: 57-70.
- Holt JG, Kriey NR, Sneath PH, Staley JT, Williams ST. *Bergey's manual of determinative bacteriology*. New York: Williams and Wilkins; 1998.
- Winnepenninckx B, Backeljau T, De Wachter R. Extraction of high molecular weight DNA from molluscs. *Trends Genet* 1993; **9**(12): 407.
- Troussellier M, Got P, Mboup M, Corbin D, Giuliano L, Cappello S, et al. Daily bacterioplankton dynamics in a sub-Saharan estuary (Senegal River, West Africa): a mesocosm study. *Aquat Microb Ecol* 2005; **40**: 13-24.
- Abo-Amer A. Biodegradation of diazinon by *Serratia marcescens* DI101 and its use in bioremediation of contaminated environment. *J Microbial Biotechnol* 2011; **21**(1): 71-80.
- Sánchez ME, Estrada IB, Martínez O, Martín-Villacorta J, Aller A, Morán A. Influence of the application of sewage sludge on the degradation of pesticides in the soil. *Chemosphere* 2004; **57**(7): 673-9.
- Beynon KI, Hutson DH, Wright AN. The metabolism and degradation of vinyl phosphate insecticides. *Residue Rev* 1973; **47**: 55-142.
- Deshpande N. Biodegradation of dimethoate-a carbamate group of organophosphorus insecticides [dissertation]. Pune: University of Pune; 2002.