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Microbial Biodegradation of Fenitrothion Pollution (Obsolete Pesticide Sumithion 50% EC)

Sherif H. Abd-Alrahman[1]*, Manal E. A. Elhalwagy[2] and A. Mostafa[3]

[1]Department of Biochemistry, College of Science, King Saud University, PO Box, 2455, Riyadh - 11451, Saudi Arabia.
[2]Department of Biochemistry, Faculty of Science for Girls, King Abdulaziz University, P.O Box 51459, Jeddah - 21453, KSA.
[3]Department of Botany and Microbiology, Collage of Science, King Saud University, P. O. 2455, Riyadh - 11451 Kingdom of Saudi Arabia.

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Accumulation of obsolete pesticides in the environment is a global problem. The scale of the problem in different countries varies depending on economic situation and social awareness. Only limited data are available on the microbial biodegradation of fenitrothion. Biodegradation of fenitrothion by different microorganisms was investigated. Six bacterial strains were isolated from an agricultural soil and found to be actively utilizedfenitrothion,as a sole source of carbonand energy. Based on their morphological and biochemical categorization, the six bacterial and fungal isolates were identified as *Pseudomonas sp.*, *Trichoderma Viride*, *Brady rhizobium sp.*, *Aspergillus niger*,and*Aspergillus flavus*. Results show that the degradation of fenitrothion by the selected microorganisms was in order *Trichoderma Viride* Ā *Aspergillus niger*Ā *Brady rhizobium sp.* Ā *Aspergillus flavus* Ā *Pseudomonas sp.*,*comparing to the control* (degradation rate in media without microorganisms). Trichoderma viridereported that the highest degradation rate ($t_{1/2}$15.64 days), followed by *Aspergillus niger*and *Brady rhizobium sp.* with $t_{1/2}$ were 22.63 and 26.58 day, respectively. While the normal degradation in the culture media was reported as 76.41 days. Our results can conclude that these three organisms can be used to degrade the obsolete fenitrothion formulation.

Key words: Fenitrothion, Biodegradation, Obsolete Pesticide.

Obsolete pesticides can shortly be defined as stocked pesticides that can no longer be used for their original purpose or any other purposes and therefore require disposal. Such pesticides can no longer be used because their use has been banned, because they have been deteriorated, or because they are not suitable for the use originally intended and cannot be used for another purpose, nor can they easily be modified to become usable[1]. Obsolete pesticides, as well as their preceding utilitarian versions, consist of active substances, that is, chemical compounds or substances which, while penetrating natural environment (waters, soil, food chain), modify and harm it, often becoming an ecological time bomb[2]. Spreading obsolete pesticides in the environment is a global problem. The scale of the problem varies depending on economic situation and social awareness. Many countries enacted strictly laws to pesticide trade and its waste management. The efforts to resolve the pesticide problem legally and effectively seem to bring first results[2]. There are yet some places in developing countries where agricultural crops are considerably contaminated with pesticide waste as Ethiopia, Tanzania, Botswana, Mali, and Madagascar[3-5]. If microorganisms are used to biodegrade sustained, toxic pollutants such as

* To whom all correspondence should be addressed.
E- mail: drsherif_hussein@yahoo.com

persistent organic pollutants (POPs) to live organisms[6-9], it is also possible to exploit them to neutralize organic obsolete pesticides. Nowadays, the use of microorganisms to biodegrade this kind of waste is almost imperceptible, as the most popular way of managing with them is thermal utilization[4]. Bacteria and fungi show the biggest capability of degrading pesticides. Bacteria, actinomycetales (special group of bacteria), and fungi show the biggest capability of degrading pesticides. The species of bacteria and soil fungi distinguishing the strongest activity in degrading pesticides are: bacteria of the genera arthobacter, bacillus, corynebacterium, flavobacterium, and pseudomonas; actinomycetales of the genera nocardia and streptomyces; and fungi *penicillium*, *aspergillus*, *fusarium*, and*Trichoderma*[10-13]. Recent study by Koliand others[14-16] pointed out in their research, the biggest Abd-Alrahman and Salem-Bekhit 331 biodegradation potential concerning bacteria Pseudomonas sp. and Nocardioides sp.

Fenitrothion [O,O-dimethyl-(3-methyl-4-nitro phenyl) phosphorothioate is an organophosphorus insecticide, which has been used widely because of its effectiveness against many insects and crustaceans, and on which there have been many studies of its metabolic effects. Under aerobic conditions, fenitrothion is biotransformed into 3-methyl-4-nitrophenol (MNP)[17, 18] amino-fenitrothion (O,O-dimethylO-(3-methyl-4-aminophenyl)-phosphorothioate)[19] and fenitrothion-oxon (O,O-dimethyl O-(3-methyl-4-nitrophenyl)-phosphate)[20]. Under anaerobic conditions, fenitrothion isbiotransformed into amino-fenitrothion[18] and MNP[21]. The major metabolites of fenitrothion under both conditions are MNPand amino-fenitrothion. Other minor metabolites havealso been reported[17, 18, 20], and theexistence of unknown metabolites has been suggested[22, 23]. Our experimental approach is to study biodegradation of fenitrothion in culture media by different five resistance isolates from Agricultural soil.

MATERIALS AND METHODS

Chemicals

Fenitrothion formulation (Sumithion 50% EC) used for fortification was obtained fromDow Agro-Sciences Ltd., Indiana, USA.The analytical standard of fenitrothion(purity 99.5 %) was purchased from Sigma-Aldrich Laborchemikalien GmbH, UK (Fig. 1).A stock solution of fenitrothion at a concentration of 10 mg ml^{-1} was prepared in dimethyl sulfoxide (DMSO). Working solutions were prepared from stock solutions. All solutions were freshly prepared. All other chemicals used were of analytical grade.

Microorganisms

Soil samples were collected from the surface 10 cm layer at the agricultural ground of contaminated field with fenitrothion. Fifty grams soil were collected from 16 different sites of the field, each sample in plastic bags and transported at once in cold storage containers to laboratory for further investigation.The soil samples were spiked with fenitrothionsolution at concentration 10 mg l^{-1} after adding water to keep the soil moisture at 50 %. Soil samples were incubated at 30 °C ±2 for three days. After the incubation the mass soil was a little air-dried, thoroughly mixed, and then sieved through 0.20 mm mesh sieve for microorganism isolation experiment and chemical analysis, respectively. The soil homogenate was inoculated in 50-ml nutrient broth (Sigma, USA) and Sabouraud dextrose broth (Difco, USA). All purified microorganisms were tested for their abilities to grow in the presence of fenitrothion and butachlor individually in (nutrient agar medium): Beef extract 3.0gm, Peptone 5.0gm, Agar-agar 20.0 gm. and Distilled water to make 1 litter (H_2O) 1.0L at pH 7.2 23. The resulting colonies were repeatedly subculture in medium containing 10 ppm fenitrothion and butachlor to confirm their pesticides-catabolising ability. Inoculated plates were incubated at (30 °C ±2) for 7days. The growth of microorganisms used for standing the toxicity of pesticide was determined and recorded as growth or inhibition. Identification and characterization of the isolates;*Pseudomonas sp.*, *Trichoderma Viride*, *Brady rhizobium sp.*, *Aspergillus niger*,and*Aspergillus flavus*were carried out on the basis of the colony morphology, biochemical characteristics and Polymerase Chain Reaction used as a supporting tool in the identification of the bacterial isolates 5.

Screening of isolates

The cultures obtained from soil enrichment were screened for fenitrothion tolerance

capacity by following the gradient plate method. The fenitrothion concentration gradient was prepared by adding a base layer of 20 ml of modified agar media without fenitrothion to a9 cm square petri plate tilted at an angle of 30. The agar was allowed to solidify at room temperature into a wedge-shaped layer. Onto the set base, another 20 ml of modified agar media with fenitrothion (10 mg l^{-1}) was poured to give afenitrothion gradient across the plate surface. Plates were incubated at 28 p C for 3 days.

Degradation experiments

Fungi isolates culture, 7 days old, were maintained at 27 ± 1°C on potato dextrose agar (PDA) medium containing (in g/l sterilized distillated water) peeled potato 200, dextrose 20 and agar 15 and served as a source of inoculum. Bacteria isolates were grown at 27 ± 1°C for 48 h on nutrient agar (NA) medium containing (in g/l sterilized distillated water) beef extract, 3; peptone, 5; agar, 15 and glucose, 2.5, then suspended in sterilized distillated water and adjusted to its optical density at A650 nm to 0.01 (10^7 cell/ml). The pH of the either PDA or NA media was adjusted to 7.0 using phosphate buffer. Bacterial suspension (1 ml) or one disc of the fungus (5 mm, diameter 1.6 × 10^6 spore/disk) was added to 250 ml Erlenmeyer flasks containing 100 ml of either NA broth medium containing (in g/l) beef extract, 3; peptone, 5; and glucose, 2.5 yielding dissolved organic carbon of 0.999 g/l or Czapek-Dox containing (in g/l) NaNO3,2;K2HPO4, 1; MgSO4.7H2O, 0.5; KCl, 0.5; FeSO4, 0.01 and sucrose, 30 yielding dissolved organic carbon of 12.62 g/l, respectively, and spiked with 0.1 ml of stock solution of fenitrothion dissolved in dimethyl sulfoxide providing a final concentration of 10 ppm. Flasks were kept at 27 ± 1°C for a period of 14 days without shaking. Aliquots (20 ml each) of each media were withdrawn by micropipette after 0, 3, 7 and 14 days of incubation and subjected to chromatographic analysis. A separate set of un-inoculated flasks was maintained as reference. All experiments were performed in triplicates.

Analysis fenitrothion residues in culture media

20 ml of culture media was collected at 0, 3, 7, 15 days, respectively during the experiment. Aqueous samples were extracted twice using 20 ml of dichloromethane. After the extraction 35 ml the organic solvent were collected in clean 50 ml falcon centrifuge tube. Water was removed from the extract using sodium sulphate anhydrous column, then the clean organic phase was dried under stem of nitrogen to dryness. The samples were kept in the freezer at -20 p C till the chromatographic analysis.

Chromatographic analysis

Agilent 6890 (USA) gas chromatography coupled with flame photometric detector (GC-FPD) was used. Separation was performed using capillary column HP-5 (30m × 0.25mm × 0.25µm). Nitrogen was used as the carrier gas at a flow rate 2ml/min. The following temperature program was employed: initial temperature of 180 ºC held for 1 min; increased at 25 ºC min-1 to 220, held for 2 minutes; yet another increase at 3 ºC min-1 to reach 245 ºC. The injector temperature was 220 ºC. The injection volume was 1 µl for all standard and samples. Data analysis was performed using Chemistation software. Calibration curves were generated by plotting peak area versus concentration. Standard calibration curves were presented excellent linearity with regression coefficient r > 0.995 with good separation and repeatability for the two tested pesticides. The calibration curve and recovery validation study were all repeated three times (n = 3).

Statistical analysis

Data were statistically evaluated by one way analysis of variance (ANOVA). Determination of the differences among means were carried out by using the least significant differences (LSD) test. All statistical analyses were done using the Statistical Package for social sciences (SPSS 16.0) program.

RESULTS AND DISCUSSION

The experiment scheme was to evaluate the resistance of isolated microorganisms to fenitrothion and determine the ability of these microorganisms to degrade fenitrothion residues in the culture media, and then the materials consisting of such microorganisms were analyzed on microorganism's biodegrading fenitrothion content to determine if bacteria and fungi strains are responsible for biodegradation. This is the first studythat examined the use of resistance isolates from agricultural soil for biodegradation of obsolete insecticide fenitrothion. Table 1, shows

Table 1. Characterization of the fenitrothion resistant isolates under condition of 10 ppm

No.	Isolates		Description
1	Bacteria	*Pseudomonas* sp.	(Gram-negative) aerobic gammaproteobacteria
2		*Brady rhizobium* sp.	(Gram-negative) soil bacteria
3	Fungi	*Aspergillus niger*	A. niger is a fungus, it is ubiquitous in soil, produce potent mycotoxins called ochratoxins
4		*Trichoderma Viride*	Fungus and a biofungicide. (Used in soil treatment for suppression of various diseases caused by fungal pathogens.)
5		*Aspergillus flavus*	Aspergillus flavus is asaprotrophicandpathogenicfungus

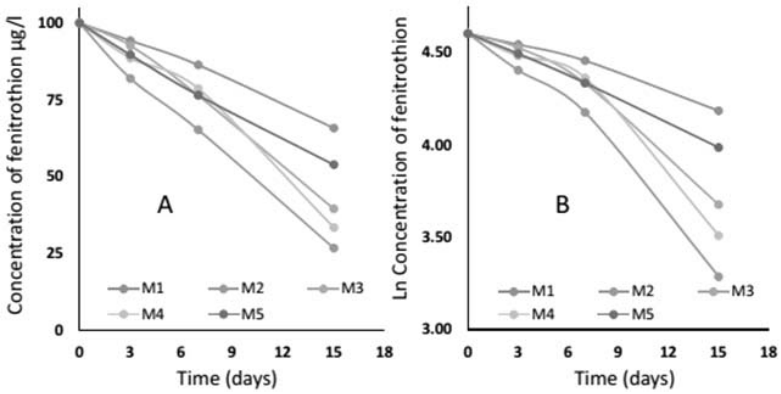

Fig. 2. Fenitrothion degradation kinetics in culture media fortified with 10ppm fenitrothion (A), and the first order kinetics (B).

the characterization of the selected isolates which used to degrade fenitrothion. The selected isolates were two bacteria (gram negative bacteria) *Pseudomonas sp., Brady rhizobium sp.* and three fungi *Aspergillus niger*, *Trichoderma Viride*, and *Aspergillus flavus*, which selected according there resistance to fenitrothion at concentration of 10 ppm. The growth results reflect that there are different resistant levels of the tested microorganisms to fenitrothion.

The biodegradation experimenthad studied the ability ofthe testedisolates to degrade fenitrothion into a basal liquid media lacking acarbon source for 14 days incubation time at 30°C±2. Results showed the microbial degradation offenitrothion as the sole carbon and energy sourcewithin 14 days incubation time at 30°C ±2. Results indicated that all oftested microorganisms can grow with the presence of fenitrothion and able to use it as carbon and phosphate source. This study report that different fenitrothion biodegradation level was obtained with *Pseudomonas sp.*, *Trichoderma Viride*, *Brady rhizobium sp.*, *Aspergillus niger*,and*Aspergillus flavus*.

Results of the biodegradation of fenitrothion as asole carbon and energy source indicated thatfenitrothion was degraded by the tested isolates from 0.0 %to 34.16, 73.22, 60.44, 66.52 and 46.06 after 14 days for *Pseudomonas sp.*, *Trichoderma Viride*, *Brady rhizobium sp.*, *Aspergillus niger*,and*Aspergillus flavus*, respectively. The half-life values were reached to76.41, 49.03, 15.64, 26.58, 22.63and 29.18 days forcontrol, *Pseudomonas sp.*, *Trichoderma Viride*, *Brady rhizobium sp.*, *Aspergillus niger*,and*Aspergillus flavus*, respectively.According to these results all of the three isolates *Trichoderma Viride, Aspergillus niger* and *Brady rhizobium* can use fenitrothion as a source of carbon (Fig 2).

Our results are in agreement with the

results of[24-26], who examined the role of microorganisms in the degradation of organophosphorus pesticides in soil. Resultsshowed that fenitrothion as organophosphoruspesticide, was biodegradable bythe co metabolic activities of soil microorganisms.Also interestingly results indicated the importantrole of nitrogen fixing such as Brady rhizobiumsp. in the degradation of fenitrothion as the solecarbon source.

During periodic cultivation, representative samples were taken approximately 0, 3, 7 and 14 days. Results show that the strains, *Trichoderma Viride, Aspergillus niger* and*Brady rhizobium*quickly degraded fenitrothion reached nearly 73.22, 66.52 and 60.44%, respectively. Figure 2 shows the degradation rate of fenitrothion by all tested microorganisms.

Fenitrothion residue analysis proved that the strain T. viride as a fungus of the Trichoderma genus was frequently recognized as good at fenitrothion biodegradation. Although the microbial degradation of fenitrothion is too weak but Trichoderma showed good ability to degrade fenitrothion which might be due to the mycelium of Trichoderma which can produce a wide variety of enzymes, including cellulases (degrading cellulose) and chitinases (degrading chitin), so it can grow directly on wood and can be a parasite of other fungi because the cell walls of fungi are primarily composed of chitin, a polymer of n-acetyl-glucosamine.

Results obtained from this work can be compared to the results of many other works concerning pesticides biodegradation of organophosphorous pesticides using microorganisms as well as with the results of works concerning mechanisms of active pesticides degradation. However, for our knowledge there is only very few data available in the research database regarding the use of biodegradation to utilize obsolete pesticides.

The mineralization rate of the herbicides depends on environmental factors, which was proved by Kodama, 2001[27].Changing the substrate reaction and incubationtemperature, he determined optimal parameters ofsimazine biodegradation for Penicillium steckii and Moraxellaovis. In the case of fungi, the best were: pH 7 to 8, 30°C, and traces of glucose and yeast extract insubstrate (over 50% subsidence of simazine in 5 days); in the case of bacteria, it was pH 5 and 35°C. Nearly 100% of simazine was degraded after 40 days of incubation of mixed bacteria culture.

Recovered and removal of fenitrothion from media inoculated withselected microorganisms

The recovered amount of fenitrothion significantly declined from the initial concentration with increasing the incubation period in the media amended with the tested microorganisms, while media without any amendment (i.e., un-inoculated control) showed less dissipation of fenitrothion (Fig 2).

In the present study, fenitrothion was used at concentration of 10 ppm (Initial concentration) without inhibitory effects to either bacteria or fungi (data not shown). In normal agriculture concentration fenitrothion did not exhibit measurable effect on soil bacteria populations[28]. The culture of Aspergillus niger could tolerate 400 ppm of technical grade of endosulfan[29]. On the other hand, complete degradation of DDT at concentrations up to 15 ppm in flasks, with shaking, had been achieved but inhibitory effects were observed at 50 ppm[30].

Kinetic studies

A biphasic model was assumed in order to carry out the statistical study of the loss of fenitrothion according to the Eq.(1).

$$R = A_0\, e^{-at} + B_0\, e^{-bt}$$

Where R is the recovered amount of fenitrothion at t days, A_0 and B_0 are the concentrations of DCF at t= 0 and α and β are the disappearance rate constants for the first and second phase model, respectively. The half-life ($t_{1/2}$) of the exponential decay was calculated according to the Eq. (2)

$$t_{1/2} = \frac{2.303 \log 2}{rate\ constant}$$

The biphasic model is characterized by a rapid phase which appears to be over in a few hours or days, and a much slower phase which may continue over weeks or even months[31, 32]. The remaining residues are often quite resistant to degradation[33]. The data indicated that there was a

faster rate of fenitrothion degradation was follow the first order kinetics as shown in figure2. This is clearly reflected in the $t_{1/2}$values, where the half-lives of fenitrothion were 15.64, 22.63, 26.58, 29.18, and 49.03 days for *Trichoderma Viride, Aspergillus niger*, *Brady rhizobium sp.,Aspergillus flavus*, and *Pseudomonas sp.*, respectively. While $t_{1/2}$ of fenitrothion in control media (non-treated with fenitrothion) was 76.41 days.

This finding are in accordance with many investigators who reported that the kinetics of pesticides degradation in soil is commonly biphasic with a very rapid degradation rate at the beginning followed by a very slow prolonged dissipation[31, 32]. The relative importance of the phases depends on the availability of the pollutants, hydrophobicity and affinity for organic matter. Fenitrothion was found to be more easily degradable than DDT and other organochlorine insecticides[34] showing the lowest accumulation hazard. Indeed, the trichloromethyl group of fenitrothion is extraordinary susceptible to carbon–carbon bond cleavage to form 4,4-0-dichlorobenzophenone[14-36].

In our experiment fenitrothion was detected in the culture media using the GC-FPD during the biodegradation of fenitrothion either by bacteria or fungi. However, in some cases the degradation of other organochlorine pesticides such as lindane has also been successfully conducted under aerobic conditions by white-rot fungi[37].

CONCLUSION

Degradation of fenitrothion proceeded rapidly in pure media amended with *Trichoderma Viride*, *Aspergillus niger* and *Brady rhizobium*, with t1/2 values of 15.64, 22.63, 26.58 days compared with 76.41 days. The relatively fast rate of fenitrothion degradation may be attributed to the adaptation of the tested microorganisms to degrade fenitrothion. During the incubation of fenitrothion with these microorganisms, no known intermediate or dead-end product could be detected using GC-FPD. These results demonstrate that fungi and bacteria can reduce the persistence of fenitrothion in liquid media culture. Our results can conclude that these three organisms can be used to degrade the obsolete fenitrothion formulation.

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