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Bioremediation of Diazinon Pesticide from Aqueous Solution by Fungal-Strains Isolated from Wastewater

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Abstract: The degradation process of diazinon one of organophosphorus pesticides was investigated at different concentrations and incubation periods by fungal species; *Rhizopus nodosus* (F1)*, Aspergillus fumigatus* (F2) and *Penciillium Citreonigum* (F3) which were isolated from wastewater. Diazinon residues were quantified at consecutive intervals during incubation period of 21 days in liquid medium inoculated with each fungal species and with control samples. The pesticide and their metabolite were extracted from samples and subsequently the extracts were analyzed by GC/MS. The observed results indicated that F1, F2 and F3 had high ability to degrade diazinon with removal efficiencies 91.1, 76.4 and 72.2% respectively. The studied fungal species F1, F2 and F3were succeeded to reduce the diazinon RL_{50} to 7.7, 10.2 and 12.1 days respectively comparing with control sample 87.8 days. 2-Isopropyl-4-methyl-6-hydroxypyrimidine (IMHP) was detected in incubated samples with the three studied fungal strains and identified as main product of biodegradation process of diazinon. We could conclude that the fungal species *Rhizopus nodosus, Aspergillus fumigatus* and *Penciillium Citreonigum* have the capability to dissipate diazinon pesticide with high efficiency.

Key words: Biodegradation · Diazinon · Pesticides · Fungi · Wastewater

INTRODUCTION

Pesticides are used in conventional agriculture to ensure the quality and yield of the crops by controlling different pests while their intensive use is also a source of environmental pollution [1, 2]. The degradation of pesticides is a major ecological problem [3]. The hydrophobic pesticides adsorbed and retained on soil particles while the water soluble pesticides enter surface and groundwater bodies through percolation, runoff and drainage [4-6]. Diazinon [O,O-diethyl O-(2-isopropyl-6-methylpyrimidin-4-yl) thiophosphate] is a broad spectrum insecticide which is considered as one of the most frequently used organophosphorus pesticides [7,8]. Diazinon has been broadly utilized to control soil and plants insects on a wide range of crops such as rice, fruits, grapes, sugarcane, corn and potatoes. Diazinon is additionally utilized to control mange bugs, ticks, lice, biting flies on sheep, cows, goats and horses [9, 10]. Diazinon entered to the surface water and soil is subjected to volatilization, hydrolysis, photolysis and biodegradation. The half-life of diazinon in water is short, it can be extended from 70 h to 12 weeks depending on pH, sunlight, temperature and the presence of microorganism [11].

The fate of diazinon pesticide in the aquatic environment was already explored [12-15]. Hydrolysis products have been distinguished as 2-isopropyl-6-methyl-pyrimidin-4-ol (IMP) and diethyl thiophosphate as illustrated in Fig. 1. Whereas photolysis studies of diazinon in soil aqueous suspensions under UV light have appeared that the most transformation product is its oxygen analogue, diethyl 2-isopropyl-6-methylpyrimidin-4-yl phosphate (diazoxon), in which S atom has been replaced by an O atom. Diazinon transformation products are more polar than the parent compound, they may be consequently more water soluble, more mobile and have a greater potential to leach from soil [16].

The use of microbial biodegradation for removal of diazinon and other organophosphorus pesticides from the environment has several advantages including the large variety of microbes available, high mutation speed and convenience in culturing [17]. Also, microbial methods are considered cost-effective and environment-friendly

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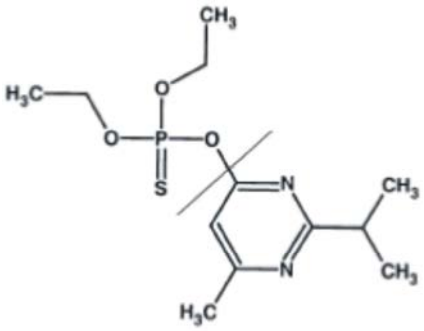

Fig. 1: Molecular structure of the diazinon pesticide

[18-20]. In this regard, numerous studies on bacteria [21-26], fungi [27-32], actinomycetes [33] yeast [34] and microalgae [35] have been carried for biodegradation of pesticides.

The purpose of this study was to isolate the fungi strains, characterize their degradation potential and their use in bioremediation of diazinon-contaminated water.

MATERIALS AND METHODS

Monitoring of the Organophosphorus Pesticides in Drainage

Study Area: The area of study is located in southern part of El-Kalubia Governorate. El-Kalubia is one of Lower Egypt governorates. It has a triangular shape with a base towards the south and top to the north. Geographically it lies between latitudes 31°5′ and 31°25′ N and longitudes 30°10′ and 30°40′ E. and is estimated to have an area of 441.5 km^2. Fig. 2 shows the location of the studied area. Agricultural wastewater samples were collected from open agricultural drains located in Egypt, at El-Kalubia governorate as shown in Table 1.

Table 1: Locations of water samples

Name of drains	Drain Code	No. of Sampling sites
Qalioubiya main drain	QB_D	3
Belbis drain	BL_D	3
Sheben El-Qanatter drain	SH_D	3
Mostorod drain	MS_D	3
Abo-Zaibl drain	AZ_D	3
Tanan drain	TN_D	3

Sample Collection: Eighteen water samples were collected from six agricultural drains at El-Kalubia Governorate as shown in Table 1. Samples were collected and transported to the laboratory for analysis according to standard methods for examination of water and wastewater [36].

Duplicate samples were collected in one-liter amber glass bottles at 50cm below water level. The bottles were covered with screw caps and the samples were immediately transported to the laboratory for analysis. Water samples were filtered to remove sand and debris.

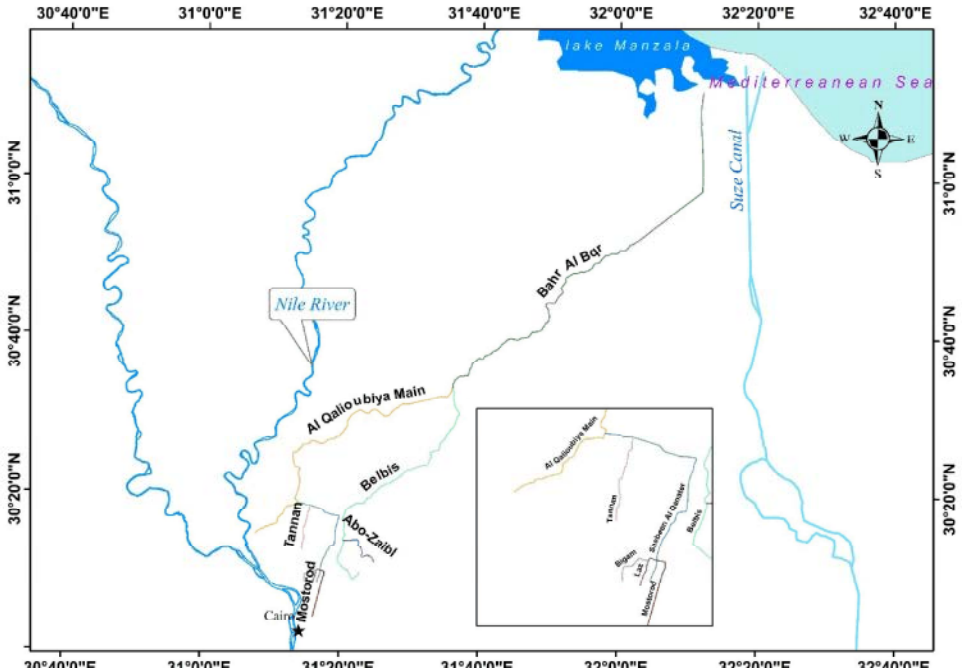

Fig. 2: Location map of the studied area

Chemicals and Reagents: The solvents used were hexane, acetone, ethyl acetate and methanol (HPLC grade, Fisher Chemicals UK). Stock Pesticide standard solution of concentration of 1000 µg/ml, (M-622), was purchased from Accustandard as mixture for organophosphorus pesticides. Other necessary materials, anhydrous sodium sulfate extra pure and glass wool were purchased from Fisher chemicals and fungi media was purchased from Oxoid limited-England. Deionized water was produced from a Milli-Q system (Millipore, Watford).

Fungi Isolation and Identification: Isolation of fungi colonies from wastewater, serial dilution followed by pour plating was done. Three samples were prepared after serial dilution to 1:10, 1:100, 1:1000, 1:10000 and from each sample one ml sample is poured into a petri plate then 30 ml of potato dextrose agar medium (PDA) was added, mixed and incubated at 28°C±2°C for 7 days until the sporulation of fungal colonies occurred. Colonies were picked up and transferred to the same growth medium. The selected species purified fungi were verified and identified [37]. The morphological determination of the isolated fungal strains was identified using Lacto-phenol cotton blue staining.

Fungal Biodegradation of Diazinon Pesticide: Isolates were inoculated into petri plates containing 30 ml of pure cultures which were grown in mineral salt liquid medium (dipotassium orthophosphate 0.2 g/l (K_2HPO_4), potassium dihydrogen orthophosphate 0.8 g/l (KH_2PO_4), magnesium sulfate 0.2 g/l ($MgSO_4$), calcium chloride 0.1g/l, ($CaCl_2$-$6H_2O$), ammonium sulfate-0.1 g/l[$(NH_4)_2SO_4$], yeast extract 0.2 g/l and distilled water one liter, pH-7.6 glucose 3 g/l).10 ppm of diazinon pesticide was added and flasks were incubated at 28°C±2°C on rotary shaker at 120rpm. After 7 days' incubation the ability of fungal strains to degrade diazinon pesticide were tested through quantitative analysis of pesticide residues using GC-MS. Control sample was carried out under the same conditions. Factors affecting the degradation process like initial concentration of pesticide, incubation time were studied for isolates with high degradation efficiencies.

Residues Extraction and Clean up Procedure: Solid Phase Extraction (SPE) technique was used for extraction of diazinon residues from the liquid medium after volume modifications of the procedure mentioned by López [38]. C_{18} Solid Phase Extraction disks (47 mm C_{18} Empore) were purchased from 3M (St. Paul, MN, USA). Millipore filtration apparatus was used for the SPE of samples. C18 disk was conditioned with ethyl acetate (5 mL) followed by methanol (5 mL) and ultrapure water (5 mL) at rate of ≈ 3 mL/ min, without allowing the cartridge to dry out. The aqueous sample (15 mL) was loaded on and passed through the cartridge at rate of ≈1 mL/ min (samples were filtered before loading to remove suspended and insoluble materials). Cartridge was dried by nitrogen stream (purity 99.999%) over surface for 2 min. Adsorbed pesticide was eluted by ethyl acetate (5 mL), treated with anhydrous sodium sulfate to remove moisture and finally the extract was adjusted to a final volume of 10 ml ready for analysis using gas chromatography.

Gas Chromatographic Analysis: Agilent 7890A gas chromatography equipped with 5975mass spectrometry detection system and HP-5MS capillary column (30m× 320µm × 0.25µm) was used for pesticide residue analysis. Carrier gas was He (>99.999%) at flow rate of 1.0 mL/min., oven was programmed at 80°C for 2 min., ramped at 10°C/min to 280°C and hold for 8 minutes. samples (final extract),1ul volumes, were injected in split/splitless injector operated at 250°C in splitless mode. Mass detector operation condition was: ion source, mass analyzer and transfer line temperatures were 230, 150 and 300°C, respectively. Mass spectrometry was operated firstly on full scan mode with mass range 50-350 m/z then operated on selected ion monitoring (SIM) mode in which insecticide was identified by retention time and specific ions and quantified by the external standard method [39-40].

Kinetic Studies: The degradation rate of diazinon was calculated mathematically according to Timme [41] who showed that degradation behavior of pesticide residues could be described mathematically as a pseudo-first order reaction, rate of degradation (K) could be calculated using common logarithms from the following equation:-

$$\log R = \log R_0 - 0.434/Kt$$

R_0: Residue level at the initial time (zero time),
R: Residue level at interval in days after application.
Kt: Constant value for rate of degradation at Time in days,
K: Mean of Kt

Diazinon half-life value (RL50) was calculated mathematically according to Moye [42] from the following equation:

$$RL_{50} = Ln2/K$$

Table 2: Organophosphorus Pesticide residues in water samples collected from agricultural drainages

	Diazinon (ng/l)		Chlorpyrifos (ng/l)		Methyl- Parathion (ng/l)		Malathion (ng/l)		Dimethoate (ng/l)	
Samples Location	Average	±SD	Average	±SD	Average	±SD	Average	±SD	Average	±SD
Qalioubiya main drain	179.82	±4.7	140.12	±4.1	128.69	±4.9	128.04	±4.5	118.38	±3.5
Belbis drain	150.04	±3.2	149.17	±4.7	N.D		N.D		N.D	
Sheben El-Qanatter Drain	140.38	±4.2	132.01	±3.6	N.D		124.09	±4.1	119.98	±3.1
Mostorod drain	160.37	±6.4	N.D		112.15	±1.9	132.54	±4.2	131.35	±4.6
Abo-Zaibl drain	N.D		113.26	±2.1	N.D		N.D		N.D	
Tanan drain	112.07	±1.7	N.D		N.D		113.05	±2.2	119.62	±3.6

Table 3: Diazinon residues after incubation with fungal strains for 7 days

Fungal strain	Diazinon initial conc. µg/ml	Diazinon residue Conc. µg/ml	% Removal
F1	10	2.18±1.57	91.1
F2	10	4.34±1.4	76.4
F3	10	4.89±1.23	72.2
Control	10	9.44±0.45	5.6

RESULTS AND DISCUSSION

Organophosphorus Pesticides Residues in Agricultural Drains at El_Qalubya Governorate: Analysis results of organophosphorus (OPs) pesticides using (GC\MS) are presented in Table 2. Among the studied organophosphorus pesticides, Diazinon was detected in five sites; Qalioubiya main, Belbis drain, Sheben El-Qanatter drain, Mostorod drain and Tanan drain with concentrations ranged from 112.07±1.7 to 179.82±4.7 ng/l, where chlorpyrifos was detected in 4 sites with Qaluob main drain, Sheben El-Qanatter drain, Mostorod drain and Tanan drain concentrations ranged from113.26±2.4 to 149.17±4.6ng/l. On the other hand, malathion was detected in four sites; Al Qalioubiya main. Sheben El-Qanatter drain, Mostorod drain and Abo-Zaibl drain, with concentrations ranged from 113.05±2.2 to 132.54 ng/l, whereas Methyl-Parathion was detected in two sites; Al Qalioubiya main Mostorod drain, with concentrations ranged from 112.15±1.9 to 128.69±4.9 ng/l. The frequent high concentrations of pesticides detected in the current study are probably due to the extensive utilization of pesticides in the agricultural fields. In this perspective, the drainage of water from agricultural fields into irrigation channels poses a critical source of risk for contamination by pesticides in the broader water environment [43].

Diazinon was the most frequently detected pesticide since it was detected in more than 80% of the studied drainage water samples. Accordingly, this pesticide was selected for conducting this research work.

Diazinon Residues: A total of 12 fungal strains were screened from drain water effluent contaminated with diazinon pesticide samples. From these 12 fungal strains 3 strains were selected (*Rhizopus nodosus* (F1), *Aspergillus fumigatus* (F2) *and Penciillium Citreonigum* (F3) based on the primary degradation efficiencies identification through exposing the twelve stains to aqueous solution of 10 mg/L diazinon for 7 days and observe their removal efficiencies through quantitative analysis of diazinon residues using GC-MS. Table 3 represents the results of selected fungal strains F1, F2 and F3 that have the highest removal efficiencies of diazinon pesticide 91.1, 76.4 and 72.2 % respectively compared with a control sample without fungi, The selected strains were used for further studies.

Effect of Different Concentrations of Diazinon on Fungal Degradation: The medium was supplemented with diazinon at different concentration levels from 10 to 100 mg/l with a control sample without fungi and incubated at 28±2°C for 21 days. The results revealed that fungal bioremediation of diazinon decreased with increasing initial concentrations of insecticides as shown in Fig. (3). The three selected fungal strains F1, F2 and F3 showed high ability to degrade concentrations of diazinon with removal percent more than 80% till the concentration level of 35 mg/l with exception of fungi strains F3 which record a degradation rate of 65% at this level of concentration. As shown in Fig. (3), for higher concentration levels the degradation percent decreased to its minimum at concentration level of 100 mg/l to reach 46.8, 34.7 and 32.8% for the fungal strains F1, F2 and F3 respectively, all results are compared with a control sample which show almost constant degradation 20% for all concentration levels without any fungal strains.

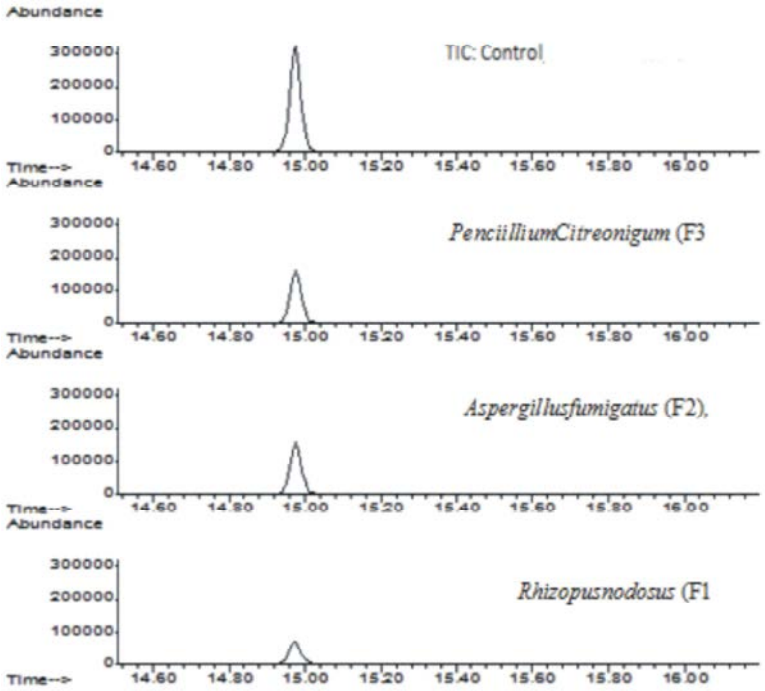

Fig. 3: An overlay of Gas chromatography/mass spectrometry chromatograms of the diazinon residues after incubation with three fungi species compared with control sample

Table 4: Effect of incubation time on diazinon degradation percent by three fungal strains F1, F2 and F3 compared with control sample

	Initial Conc.							
	Control		F1		F2		F3	
Time in days	Residues µg/ml 35µg/ml	% Removal ---	Residues µg/ml 35 µg/ml	% Removal ----	Residues µg/ml 35 µg/ml	% Removal ----	Residues µg/ml 35 µg/ml	% Removal -----
1	34.86	0.4	31.101	11.14	31.7345	9.33	32.326	7.64
3	34.54	1.3	29.1865	16.61	29.8515	14.71	30.485	12.9
7	32.48	7.2	23.786	32.04	24.955	28.7	27.125	22.5
14	30.59	12.6	8.89	74.6	13.3	62	14.455	58.7
21	27.93	20.2	3.115	91.1	8.12	76.8	9.73	72.2
K	0.0079		0.0894		0.0676		0.0572	
P value			0.046		0.062		0.085	
RL_{50} (days)	87.8		7.7		10.2		12.1	

On the other hand the effect of incubation time was studied by measuring diazinon residues concentration after incubation with each species of the three studied fungal strains at diazinon concentration level 35 mg/l for 21 day and subsamples were taken periodically at intervals time from 1 hour to 21 day and analysed for diazinon level using GC-MS and compared with control sample. The results showed that degradation of diazinon increased with increasing incubation time as shown in Table (4). Fungal strain F1 gave the highest degradation percent 91.1%, F2 strains came second by about 76.4%, while F3 strains recorded the lowest degradation 72.2%, all after 21 days of incubation.

Results of this study clearly demonstrated that the *Rhizopus nodosus* (F1), *Aspergillus fumigatus* (F2) and *Penciillium Citreonigum* (F3) had a significant influence on diazinon degradation rate and the fungal strain F1 was the most effective strain for diazinon degradation, followed by F2 and F3. Calculated half-life values (RL_{50}) for diazinon inoculated with Fungal stains F1, F2 and F3 is 7.7, 10.2 and 12.1 days respectively compared with the value for control sample 87.7 days as shown in Table (4).

Diazinon Degradation Product: In this research paper biodegradation of diazinon was followed by GC-MS which showed that 2-Isopropyl-4-methyl-6-hydroxypyrimidine

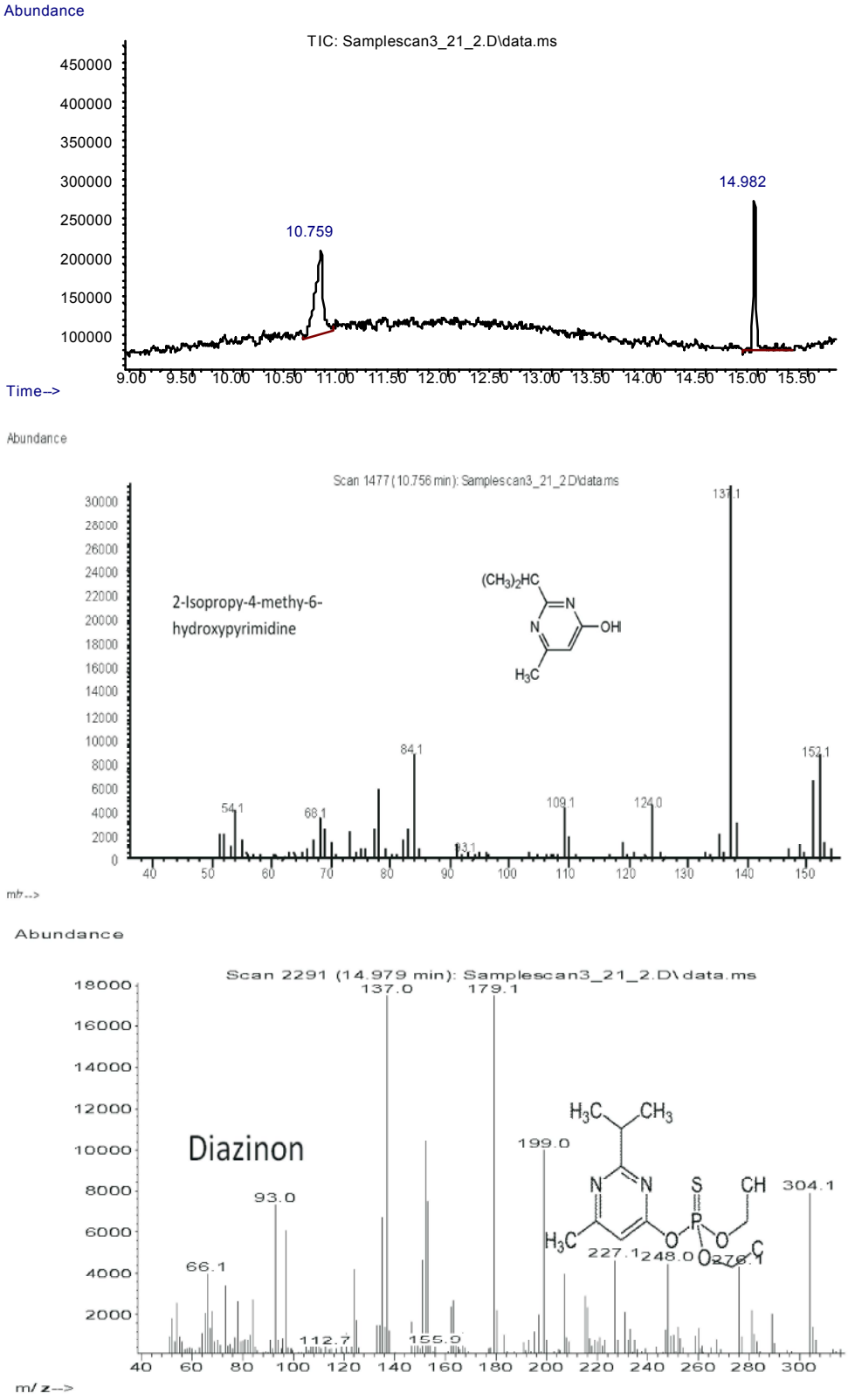

Fig. 4: GC-MS chromatogram for a sample incubated with fungal strain and mass spectra of diazinon and degradation product (IMHP) identified by comparison with known standards

(IMHP) was detected in incubated samples with the three studied fungal strains (F1, F2 and F3) and not appeared with the control sample. Fig. (4) shows the appearance of IMHP as a tailed and relatively broad peak at 10.75 min while the retention time for diazinon was 14.98 min. Structural data were obtained from the EI fragmentation patterns and library searching.

Analysis of the fragment ions of diazinon (MW 304 Kda) the base ion m/z 179, the ions of m/z 137 and 152 in the mass spectrum gives information regarding the structures of the characteristic ions of the pyrimidine moiety. While for IMHP (MW 152KDa) the base peak at m/z 137 in the mass spectrum Fig. (4) corresponded to the pyrimidine species produced following the loss of a methyl radical from the molecular ion of m/z 152. The fragmentation pattern of the IMHP ion of m/z 137 contained the product ion of m/z 84 and was identical to the corresponding MS fragmentation pattern for diazinon. IMHP is the Degradation product of diazinon formed through the cleavage of P=O(pyrimidine group) bond, found in water, soil, plants and animals that has been reported by many other scientists [44-48].

CONCLUSIONS

In the present study degradation of diazinon pesticide was studied in aqueous solution and darkness, over a period of 21 days using fungal strains isolated from wastewater. Degradation batch experiments were carried in mineral salt medium supplemented with diazinon as the sole source of carbon. Results of this study clearly demonstrated that *Rhizopus nodosus*(F), *Aspergillus fumigatus* (F2) and *Penciillium Citreonigum* (F3) had a significant influence on diazinon degradation rate. Calculated half-life time values (RL_{50}) for diazinon inoculated with Fungal stains F1, F2 and F3 were 7.7, 10.2 and 12.1 days respectively compared with the value for control sample 87.7 days. During the degradation of diazinon, its hydrolytic metabolite 2-Isopropyl-4-methyl-6-hydroxypyrimidine (IMHP) is formed. The monitoring of diazinon degradation and formation of it metabolite IMHP was done using GC-MS, by comparison with known standards. Finally, it could be concluded that, this paper study diazinon biodegradation using fungal strains under the laboratory conditions, is hoped to be a useful step for further study on a larger scale.

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